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SHORT COMMUNICATION

Leukemia with distinct phenotypes in transgenic mice expressing PML/RARα, PLZF/RARα or NPM/RARα

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Recurrent chromosomal translocations involving the *RARα* locus on chromosome 17 are the hallmark of acute promyelocytic leukemia (APL). The *RARα* gene fuses to variable partners (*PML*, *PLZF*, *NPM*, *NuMA* and *STAT5B*: *X* genes) leading to the expression of APL-specific fusion proteins with identical RARα moieties. To analyse whether the variable X moiety could affect the activity of the fusion protein *in vivo*, we generated and characterized, on a comparative basis, NPM/RARα transgenic mice (TM) in which the fusion gene is expressed under the control of a *human Cathepsin G* (*hCG*) minigene. We compared the features of the leukemia observed in these TM with those in hCG-PML/RARα and hCG-PLZF/RARα TM. In all three transgenic models, leukemia developed after a variably long latency, with variable penetrance. However, the three leukemias displayed distinct cytomorphological features. hCG-NPM/RARα leukemic cells resembled monoblasts. This phenotype contrasts with what was observed in the hCG-PML/RARα TM model in which the leukemic phase was characterized by the proliferation of promyelocytic blasts. Similarly, hCG-PLZF/RARα TM displayed a different phenotype where terminally differentiated myeloid cells predominated. Importantly, the NPM/RARα oncoprotein was found to localize in the nucleolus, unlike PML/RARα and PLZF/RARα, thus possibly interfering with the normal function of NPM. Similarly to what was observed in human APL patients, we found that NPM/RARα and PML/RARα, but not PLZF/RARα leukemia, was responsive to all-*trans* retinoic acid (ATRA) or As_2O_3 treatments. Taken together, our results underscore the critical relevance of the X moiety in dictating the biology of the disease and the activity of the APL fusion oncoprotein.
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The chromosomal translocations t(15;17)(q22;q21), t(11;17)(q23;q21) and t(5;17)(q35;q21) are characteristic of acute promyelocytic leukemia (APL) and lead to the fusion of the *RARα* gene to the *PML*, *PLZF* and *NPM* gene (hereafter referred to as *X* genes), respectively (Brunning *et al*., 2001; Rego and Pandolfi, 2001; Sirulnik *et al*., 2003). In the fusion proteins encoded by these hybrid genes, identical RARα moieties are fused to structurally unrelated X moieties (Rego and Pandolfi, 2001; Sirulnik *et al*., 2003). The X/RARα oncoproteins have been proposed to act in a dominant-negative fashion to suppress the physiological function of retinoic acid and X-dependent pathways (Piazza *et al*., 2001). Several groups, including our own, have generated PML/RARα and PLZF/RARα transgenic mice (TM) in which the respective fusion genes were placed under the control of *human Cathepsin G* (*hCG*) regulatory sequences, thus being expressed in early myeloid progenitors (Grisolano *et al*., 1997; He *et al*., 1997, 1998; Cheng *et al*., 1999). In both TM models, the development of leukemia was observed after a long pre-leukemic phase, characterized by myeloproliferation, strongly suggesting that additional genetic events are necessary for full-blown leukemogenesis (Grisolano *et al*., 1997; He *et al*., 1997, 1998; Cheng *et al*., 1999). Several studies have emphasized the importance of the deregulation of the X pathways in APL pathogenesis: (i) PML haploinsufficiency in hCG-PML/RARα TM increased the frequency and accelerated the onset of leukemia (Rego *et al*., 2001); (ii) PLZF inactivation caused a change of the cytomorphological features detected in hCG-PLZF/RARα TM leukemia from mature into immature myeloid cells (i.e. triggered a block of differentiation) (He *et al*., 2000); (iii) the expression of PML/RARα disrupts the localization of wild-type PML, causing it to relocalize from discrete nuclear structures, the PML nuclear bodies, into microspeckled aberrant structures (Dyck *et al*., 1994; Piazza *et al*., 2001). PML/RARα also disrupts the

function of cytoplasmic PML, thus impairing TGF-β signaling (Lin *et al*., 2004). To determine the functional relevance of the X partners of RARα, we have generated hCG-NPM/RARα TM, and compared the incidence, latency, morphological and immunophenotypic features of leukemia in the three models: hCG-PML/RARα, hCG-PLZF/RARα and hCG-NPM/RARα.

In order to compare the three transgenic models, the same hCG minivector, mouse strain (CBA/BL6), and Transgenic Facility were used. The generation of hCG-PML/RARα and hCG-PLZF/RARα were described elsewhere (He *et al*., 1997, 1998). A total of 66 hCG-NPM/RARα, 124 hCG-PML/RARα and 38 hCG-PLZF/RARα TM, as well as non-transgenic littermates (wild-type controls, $n=30$) had their automated and differential counts of peripheral blood (PB) monitored monthly. Animals presenting palpable tumors, anemia (Hemoglobin$\leqslant$12 g/dl), thrombocytopenia (Platelets$\leqslant$500.000/μl) or abnormal cells in PB smears were killed and spleen, liver lymphonode (LN), bone marrow (BM) were collected for analysis. In order to characterize the hematopoiesis in the hCG-NPM/RARα TM, two TM and two littermate controls were killed every 3 months starting from the third month of age, and morphological analysis of PB, BM, spleen, LN and liver was carried out.

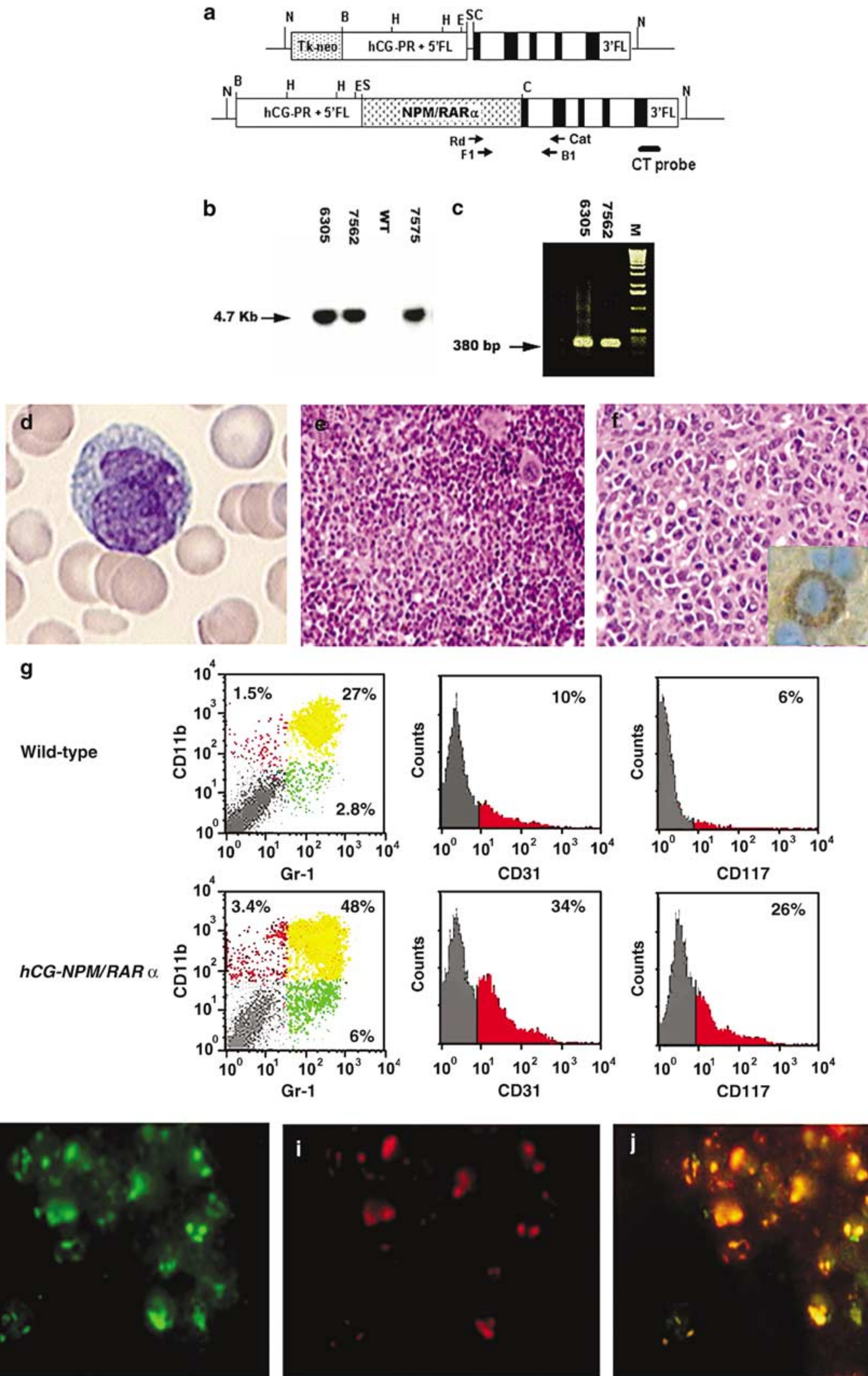
Follow-up of hCG-NPM/RARα TM revealed that they developed leukemia with distinct and unexpected features. Table 1 summarizes, in a comparative manner, the main hematological features of leukemia presented by hCG-PML/RARα; hCG-PLZF/RARα and hCG-NPM/RARα TM. In all three models, leukemia was detected after a long latency (in the case of hCG-NPM/RARα TM was detected only after the first year of life). Leukemia was diagnosed with similar frequency in both hCG-NPM/RARα transgenic lines (five out of 40 (12.5%) and three out of 26 (11.5%) mice from the 7562 and 6305 lines, respectively), as well as in hCG-PML/RARα TM. By contrast, in hCG-PLZF/RARα leukemia was fully penetrant, but occurred again after a long latency. Thus, these fusion proteins are necessary, but not sufficient for leukemogenesis in the mouse. Hepato- and splenomegaly was invariably detected in leukemic TM, regardless of which *X/RARα* gene is expressed. Leukemia was characterized by anemia, leukocytosis and thrombocytopenia in the three transgenic models (Table 1), but leukocyte counts were significantly lower ($P<0.05$) in NPM/RARα. However, the cytomorphological features of these leukemias were strikingly different in the various models. In hCG-PML/RARα the leukemic cells resemble promyelocytes in PB and BM, being the closest to human APL, whereas in hCG-PLZF/RARα, no block of myeloid of differentiation was detected with large numbers of mature granulocytes in BM. Although an increase in mature granulocytes was also detected in PB, the difference was not significant ($P=0.16$). This leukemia has many of the features of a chronic myelogenous leukemia (CML), albeit being rapidly fatal. hCG-PLZF/RARα TM generated by Cheng *et al*. (1999) presented the same cytomorphological features described here, nevertheless their model was not fully penetrant and only 5/51 founder mice developed leukemia. In contrast with the APL-like and the CML-like phenotype developed by hCG-PML/RARα and hCG-PLZF/RARα TM, respectively, leukemic hCG-NPM/RARα presented a small

Table 1 Clinical and hematological features of leukemia in hCG-NPM/RARα, hCG-PML/RARα and hCG-PLZF/RARα transgenic mouse models

	Genotype	*NPM/RARα*	*PML/RARα*	*PLZF/RARα*	*Wild-type*
	Frequency of leukemia	8/66 (12%)	12/124 (10%)	36/38 (95%)	0/30 (0%)
	Age at diagnosis (months)	15.8±4	12–15	6–18	NA
	Hepato-splenomegaly	Present	Present	Present	Absent
Blood	Hemoglobin level (g/dl)	8.7±2	9.9±3.8	9.1±2.3	16.3±1.1
	White blood cell counts (×10³/μl)	28.8±4.1*	137.4±149.7	286.9±158.4	10.7±3.4
	Platelet counts (×10³/μl)	498±135	245.6±192	398.8±206	1250±230
	Segmented+Bands (%)	57.1±8.1	69±19	88.9±15.3	14±8
	Promyelocytes+Blasts (%)	0	17.7±10**	1.5±1.0	0
	Monoblasts (%)	3.2±1.4*	0	0	0
	Myeloid/Lymphoid ratio	6.7±4.2	7.75±5.7	14.8±6.1	0.27±0.12
Bone Marrow	Segmented+Bands (%)	38.9±13.8	10.9±9.7	71.0±14.4***	30.8±13.1
	Metamyelocytes+Myelocytes (%)	20.4±3.2	31.1±11.2	29±13.6	10.2±5.5
	Promyelocytes+Blasts (%)	5.3±2	51.9±14.9**	11.7±7.7	3±1
	Monoblasts (%)	28.2±5*	<3	<3	<3
	Myeloid/Lymphoid ratio	31±9.5	42.2±14.5	45±19.3	3.7±2.3
	Leukemic cell immunophenotype	Mac-1⁺ Gr1⁺ CD117⁺ CD31⁺ Lysozyme⁺	Mac-1⁺ Gr1⁺ CD117⁺ CD31± Lysozyme⁻	Mac-1⁺ Gr1⁺ CD117± CD31± Lysozyme⁻	NA

Values represent mean±standard deviation (s.d.); NA – not applicable. *Significantly different ($P<0.05$) from the observed in leukemic hCG-PML/RARα and hCG-PLZF/RARα. **Significantly higher than the observed in leukemic hCG-NPM/RARα and hCG-PLZF/RARα. ***Significantly higher than the observed in leukemic hCG-PML/RARα and hCG-NPM/RARα.

percentage (3.2 ± 1.5%) of leukemic blasts with monocytoid features (Figure 1d), thus resembling an acute myelomonocytic leukemia (Table 1, Figure 1e and f). The myelomonocytic nature of leukemia was confirmed by immunophenotyping, which demonstrated that the cells were CD117$^+$, Gr-1$^+$, Mac-1$^+$ and CD31$^+$



(Figure 1g). The expression of lysozyme was confirmed by immunohistochemistry as well (Figure 1f inset). Interestingly, Cheng *et al*. (1999) also generated hCG-NPM/RARα TM, which developed leukemia at a low frequency after a long pre-leukemic phase (>410 days). However, these leukemias were described as ranging from typical APL to CML-like. It must be emphasized, however, that this conclusion was solely based on the analysis of three mice (Cheng *et al*., 1999). Furthermore, the differences between our model and those described by Cheng *et al*. may be owing to the fact that different mouse strains were employed (CBA/BL6 in the present study and C57 by Cheng *et al*.).

In hCG-NPM/RARα TM older than 12 months we invariably detected a pre-leukemic phase characterized by: (i) hepato-splenomegaly owing to infiltration by mature granulocytic and monocytoid cells and, (ii) increased myeloid:lymphoid ratio in the BM in the absence of significant changes in the PB. Regarding the existence of a long latency until the development of leukemia, hCG-NPM/RARα TM model was similar to the previously described hCG-PLZF/RARα and hCG-PML/RARα TM models. Therefore, regardless of the fusion gene additional mutagenic events appear to be required for full-blown leukemogenesis.

As PML-RARα and PLZF-RARα accumulate in discrete subnuclear structures in blasts from both human APL patients and TM (He *et al*., 1997, 1998; Ruthardt *et al*., 1998; Zhong *et al*., 2000), we wanted to determine the subcellular localization of NPM/RARα. We at first stained cells from leukemic TM utilizing an anti-RARα antibody (Ab). The fusion proteins accu-

Table 2 Cytomorphological analysis of the peripheral blood and bone marrow of nude mice transplanted with hCG-PML/RARα, hCG-NPM/RARα or hCG-PLZF/RARα leukemic cells before and after ATRA treatment

		Pretreatment			*21st day of ATRA treatment*		
		PML/RARα (n = 13)	*NPM/RARα (n = 13)*	*PLZF/RARα (n = 13)*	*PML/RARα (n = 13)*	*NPM/RARα (n = 13)*	*PLZF/RARα (n = 13)*
Blood	Hemoglobin level (g/dl)	10.2±2.1	9.7±2.7	11±1.8	13.2±1.2	14.5±2	10.1±3.1
	White blood cells (×10³/μl)	25±9.7	26.6±5.4	24.3±8.3	18.2±5.7	16.4±6.7	41.1±13
	Segmented+Bands (%)	41.4±10.7	48.8±9.8	75.6±18.2	88.2±8.2	76.2±11.3	64.8±15.6
	Blasts+Promyelocytes (%)	35.4±7.2	5.3±3.1	7.2±3	0	0	12.2±7.5
	Monoblasts (%)	0	22.4±7.4	0	0	0	0
Bone Marrow[a]	Segmented+Bands (%)	28.8±8.9	32.5±8.8	77.6±18.8	81.2±12	76.5±13.4	67.3±10.4
	Blasts+Promyelocytes (%)	65±21.9	12.2±6.7	8.7±4.8	5.2±5.2	4.8±3.2	21.1±12.1
	Monoblasts (%)	1.8±1.2	35.1±8.6	2.1±1.8	2.1±1.8	1.9±1.2	2.2±1.4

[a]BM differential counts at the 21st day represent mean±standard deviation of three mice per genotype.

Figure 1 hCG-NPM/RARα TM develop myelomonocytic leukemia. (**a**) Schematic representation of the hCG-NPM/RARα transgene. The exons in *hCG* minigene expression vector are indicated by filled boxes, the Tk-neo cassette; the *hCG* promoter, 5′ flanking region (hCG-PR + 5′ FL) and 3′ flanking region (3′ FL) are indicated. The *NPM/RARα* cDNA encoding the short isoform of the fusion protein was cloned into SalI site of the vector. The BamHI-NotI fragment depicted below was injected in eggs of Sv129 mice. Restriction endonuclease sites indicated are: N: NotI; B: BamHI; H: HindIII; C: ClaI; S: SalI; E: EcoRI. The location of CT probe (bar) used for Southern blot and primers used for RT–PCR (arrows) are shown beneath the transgene. (**b**) Southern-blot analysis of genomic tail DNA from the three hCG-NPM/RARα transgenic founders (6305; 7562 and 7575) and the wild-type (WT) control. Genomic DNA was obtained using the Puregene DNA Purification Kit (Gentra Systems Inc., Minneapolis, Minn, USA) digested with EcoRI (New England Biolabs, Beverly, MA, USA) and hybridized with CT probe, the expected 4.7 kb band demonstrates the integration of the transgene. (**c**) RT–PCR analysis detects expression of NPM/RARα mRNA in bone marrow (BM) cells of F1 progeny from 6305 and 7562 founders. Total RNA prepared from BM cell suspensions were incubated with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), and generated cDNAs were amplified by PCR in the presence of primers specific for the transgene. (**d–f**) hCG-NPM/RARα TM develop acute myelomonocytic leukemia. Magnified detail (×1000) of a leukemic cell detected in the peripheral blood smear from a hCG-NPM/RARα TM stained with Giemsa. Histological analysis of paraffin sections of BM (**e**) and spleen (**f**) from leukemic hCG-NPM/RARα TM showing the infiltration by granulocytic and monocytic immature cells (×400). Immunohistochemistry analysis with an anti-lysozyme antibody (polyclonal EC 3.2.1.17, Dako, Capenteria, CA, USA) was performed on frozen sections of spleen (**f**, inlet) using the Dako Animal Research Kit (ARK)-Peroxidase according to the manufacturer's instructions. The magnified detail (×800) shows that the leukemic cells expressed lysozyme. (**g**) Leukemic cells from hCG-NPM/RARα TM express myeloid and monocytic markers. Flow cytometry analysis was performed on BM samples of leukemic TM with the following Phycoerythrin- or Fluorescein-conjugated monoclonal antibodies: CD11b (Mac-1), Gr-1, CD117, CD31 (PECAM-1), CD45R/B220, CD3ε, CD45 (PharMingen). Flow cytometric analysis of BM cells from a wild-type control (upper panel) and a leukemic hCG-NPM/RARα TM (lower panel). The percentages of CD11b (Mac1), Gr1, CD117 (cKit) and CD31 (PECAM-1) positive cells are indicated. hCG-NPM/RARα TM displayed a higher percentage of cells expressing these four markers. (**h–j**) NPM/RARα fusion protein is localized in the nucleolus. Immunofluorescent confocal microscopy was performed on leukemic cells permeabilized and stained for RARα (**h**) and nucleolin (**j**) using a rabbit polyclonal anti-RARα antibody (a gift from Dr Pierre Chambon, Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France), and a mouse monoclonal anti-nucleolin (Medical and Biological Laboratories). For detection Texas Red- or Fluorescein (FITC)-conjugated goat anti-rabbit IgG or horse anti-mouse IgG were used as secondary antibodies. Immunofluorescent confocal microscopy analysis of dually stained leukemic cells revealed that NPM/RARα colocalizes with nucleolin (**j**). Slides were analysed in the Sloan-Kettering Institute's Confocal Microscopy Core Facility.

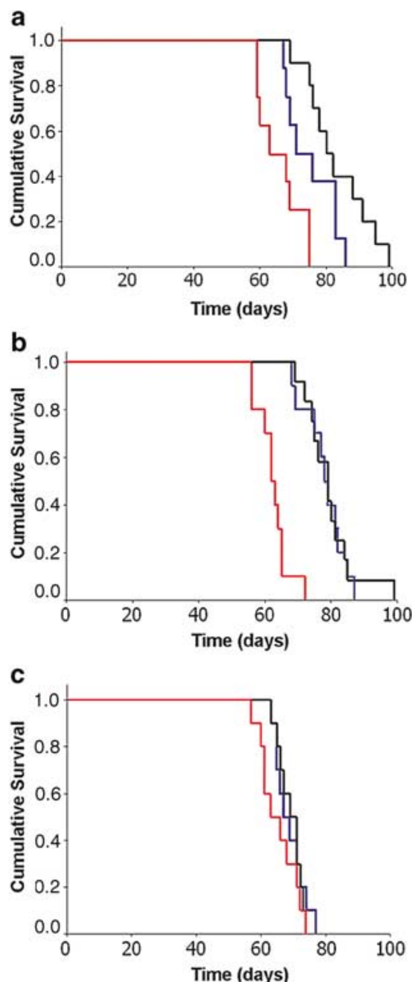


Figure 2 PML/RARα and NPM/RARα, but not PLZF/RARα leukemia, respond to ATRA and As_2O_3 treatment. A total of 117 NU/J Hfh 11[nu] mice (NM) were injected i.p. with 5×10^7 leukemic cells. The leukemic cells were obtained from the bone marrow (BM) and spleen of moribund leukemic hCG-PML/RARα, hCG-NPM/RARα or hCG-PLZF/RARα mice. Those NM that received leukemic cells harboring the same fusion genes ($n = 39$) were subdivided in three groups according to treatment: vehicle DMSO ($n = 13$), ATRA 1.5 μg/g of body weight/day ($n = 13$) or As_2O_3 5 μg/g/day ($n = 13$). Drugs were administered through an i.p. daily injection from the 25th day after transplant, and maintained for 21 consecutive days. Three mice per treatment per X/RARα fusion gene group were killed on the 21st day of treatment (46th after transplant) for cytomorphological analysis of PB and BM. The remaining 10 NM were transplanted with hCG-PML/RARα (**a**), 10 with hCG-NPM/RARα (**b**) and 10 with hCG-PLZF/RARα (**c**) leukemic cells and treated with vehicle (red line), ATRA (black line) or As_2O_3 (blue line) were analysed for cumulative survival. Survival was defined as the time (in days) from transplant until death. All animals relapsed shortly before death and leukemic infiltration was detected in BM and spleen. The survival analysis was based on Kaplan–Mier estimation and groups were compared by the log-rank test using the SPSS 9.0 software (SPSS Inc., Chicago, USA).

mulated in large nuclear structures, which were reminiscent of nucleoli, the main subcellular structure containing native NPM (Figure 1h). Indeed, co-staining with anti-nucleolin and anti-RARα Abs demonstrated that NPM-RARα did accumulate in the nucleoli (Figures 1h–j). This is in agreement with what was reported by Hummel *et al.* (2002) in COS cells overexpressing different X/RARα fusion proteins: NPM/RARα was found to accumulate predominantly in the nucleolus, whereas PML/RARα and PLZF/RARα did not (Hummel *et al.*, 2002). NPM/RARα can heterodimerize with NPM (Redner *et al.*, 2000; Piazza *et al.*, 2001; Sirulnik *et al.*, 2003). Thus, our results suggest that the fusion protein may deregulate NPM function at the nucleolus through physical interaction. NPM has been recently found mutated in a very high percent of acute myelogenous leukemia (AML), most of which present myelomonocytic (M4) or monocytic (M5) phenotype (Alcalay *et al.*, 2005; Falini *et al*, 2005; Grisendi and Pandolfi, 2005). As NPM/RARα is thought to exert a dominant-negative action on NPM, it is conceivable to speculate that the monocytic phenotype detected in hCG-NPM/RARα TM, similar to the one in AML with NPM mutations, could be determined by a loss of NPM function. NPM is implicated in control of ribosome biogenesis, genomic stability and the regulation of ARF nucleolar function, among other things (Okuda, 2002; Kurki *et al.*, 2004; Grisendi *et al.*, 2005). Therefore, the ability of NPM/RARα to interfere with NPM may contribute to the leukemic phenotype at multiple levels.

All *trans* retinoic acid (ATRA) is the mainstay of the treatment of APL (Degos, 2003). It induces leukemic cell differentiation and disease remission in patients with t(15;17)/PML/RARα or t(5;17)/NPM/RARα. Similarly, As_2O_3 has been demonstrated to be effective in the treatment of *de novo* as well as of ATRA-resistant t(15;17)/PML/RARα APL (Degos, 2003). In contrast, patients with t(11;17)/PLZF/RARα are resistant to ATRA and have a dismaying prognosis. In order to compare the response to ATRA and As_2O_3 of leukemia in hCG-PLZF/RARα, hCG-PML/RARα and hCG-NPM/RARα TM, we transplanted nude mice (NM) with leukemic cells from the different TM models as previously described (Rego *et al.*, 2000) and treated the recipients with vehicle (DMSO), ATRA or As_2O_3 for 21 consecutive days starting on the 25th day post transplant (when invariably leukemic infiltration of PB and BM was detected). Differential counts of PB were performed weekly, whereas BM differential counts were performed in cytospins at the end of treatment (three mice per treatment group of each X/RARα fusion gene) or after death. Treatment with ATRA or As_2O_3 of NM transplanted with hCG-NPM/RARα or hCG-PML/RARα leukemic cells resulted in the clearance of leukemic cells and increase of mature granulocytic cells in PB and BM (Table 2). Interestingly, we did not detect an increase of mature monocytic cells in BM of recipients of hCG-NPM/RARα cells after 21 days of treatment with ATRA, thus suggesting that the differentiation occurred along the granulocytic pathway (Table 2). In contrast, no difference was detected in the percentage of blasts and granulocytes between vehicle and ATRA-treatment in NM injected with hCG-PLZF/RARα leukemic cells (Table 2). In addition, ATRA significantly prolonged survival of PML/RARα

NM (median survival time, 95% confidence interval (95%CI) equal to 80 days, 73.8–86.2 days and 59 days, 57.9–60 days in ATRA-treated and controls, respectively, $P<0.001$) (Figure 2a) and of NPM/RARα NM (79 days, 95%CI: 74–84 days and 62 days, 95%CI: 58.9–65.1 days in ATRA-treated and controls, respectively, $P<0.001$) (Figure 2b). In contrast, PLZF-RARα NM did not respond to ATRA treatment (69 days, 95%CI: 64.8–73.1 days and 63 days, 95%CI: 55.3–70.7 days in ATRA-treated and controls, respectively, $P=0.95$) (Figure 2c). Similar results were observed with As_2O_3 treatment (Figure 2a–c), which prolonged median survival of recipients of PML/RARα cells to 71 days, (95%CI: 61.3–80.7 days, $P=0.001$) and of NPM/RARα cells to 78 days (95%CI: 74.9–81.1 days, $P<0.001$), but did not significantly change the survival of NM transplanted with PLZF/RARα cells (67 days, 95%CI: 62.3–71.6 days, $P=0.25$). Therefore, our results demonstrate that, similar to what is observed in APL patients, the X moiety of X-RARα fusion proteins determines ATRA and As_2O_3 sensitivity.

Our comparative analysis of the leukemic phenotypes observed in transgenic models expressing three different APL fusion genes lend further support to the relevance of the X moiety in determining the biology of the disease. It is important to point out that this is the first study comparing leukemogenesis induced by NPM/RARα, PML/RARα and PLZF/RARα in TM aged out at the same time in the same facility with the same genetic background. In our transgenic models, the expression of the transgene was under the control of hCG promoter, which drives expression in immature myeloid cells with particularly high levels, detected in promyelocytes (Heusel *et al*., 1993). Surprisingly, the three models developed leukemia with distinct cytomorphological features. In humans, leukemic cells harboring the t(15;17)/PML/RARα; t(11;17)/PLZF/RARα; t(5;17)/NPM/RARα may vary in morphology, but are undoubtedly classified as APL (Sainty *et al*., 2000; Brunning *et al*., 2001). The expression of the fusion protein in hCG-TM may occur in principle at later stages in hematopoiesis compared to APL patients. Therefore, the resulting phenotype may perhaps be influenced by the stage of differentiation in which the fusion gene is expressed. Nevertheless, in the case of PLZF/RARα we have previously demonstrated that the coexpression of the RARα/PLZF reciprocal product metamorphoses the CML-like phenotype into APL (He *et al*., 2000); it remains to be determined what could be the missing event in the NPM-RARα model.

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