Down-regulation of *HOXA4*, *HOXA7*, *HOXA10*, *HOXA11* and *MEIS1* during monocyte-macrophage differentiation in THP-1 cells

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Abstract. The translocation t(9;11)(p22;q23) generates the *MLL-AF9* oncogene and is commonly associated with monocytic acute myeloid leukemia (AML-M5; FAB-classification). For the oncogenicity of *MLL-AF9,* the (over)expression of several other genes, including selected *HOXA* cluster genes as well as *MEIS1* (a HOX cofactor), is required. We previously showed that the down-regulation of *MLL-AF9* expression is not obligatory for monocyte-macrophage maturation in AML-M5 cells carrying t(9;11)(p22;q23). In this study, we analyzed the expression patterns of *HOXA4*, *5*, *6*, *7*, *9*, *10* and *11* (defined as '*HOXA*-code' genes) and *MEIS1* by semiquantitative RT-PCR during the monocyte-macrophage differentiation induced by phorbol 12-myristate 13-acetate (PMA) in THP-1 cells carrying t(9;11)(p22;q23) and expressing *MLL-AF9*. The analyses were performed in THP-1 cells expressing *MLL-AF9* even after PMA treatment. The results showed that all the analyzed genes were expressed in untreated THP-1 cells. After the induction of differentiation, we observed a down-regulation of *HOXA4*, *7*, *10*, *11* and *MEIS1*, an up-regulation of *HOXA6,* and no significant variation in the expression of *HOXA5* and *9*. These data indicate that the expression of most *HOXA*-code genes, as well as *MEIS1,* could be implicated in the differentiation blockage observed in *MLL-AF9*-related leukemias.

Introduction

The most recurrent 11q23/*MLL* translocation in acute myeloid leukemia (AML) is the t(9;11)(p22;q23) translocation, which originates in the *MLL-AF9* oncogene and is mainly associated with the monocytic phenotype (AML-M5; FAB-classification) (1,2). Studies on the role of *Mll-AF9* gene fusion conducted in mouse embryonic stem cells indicate that *Mll-AF9* induces excessive myeloproliferation, but secondary tumorigenic mutations are necessary for the development of fully transformed leukemic cells (3). Gene expression profiling revealed that *Mll-AF9* expression in mouse bone marrow cells led to the (over)expression of several genes, including *Hoxa4*, *5*, *6*, *7*, *9*, *10* and *11* (defined as '*Hoxa*-code' genes) and *Meis1*, a Hox cofactor (4-6). Therefore, cooperation between *Mll-AF9*, '*Hoxa*-code' and *Meis1* gene expression seems necessary for the induction of the leukemogenetic process in mouse hematopoietic progenitors (4,5,7). Previously, we and others showed that targeted down-regulation of MLL-AF9 in a human leukemia cell line, THP-1, induced strong inhibition of cell proliferation (8,9), but did not affect the terminal differentiation of THP-1 cells (8). Successively, we reported that down-regulation of *MLL-AF9* expression is not mandatory for monocyte-macrophage maturation in AML-M5 cells carrying the translocation t(9;11)(p22;q23), hypothesizing that other genes are important for differentiation blockage in leukemogenesis induced by *MLL-AF9* (10). In this study, we analyzed the expression patterns of *HOXA4*, *5*, *6*, *7*, *9*, *10*, *11* and *MEIS1* by semiquantitative RT-PCR during the monocyte-macrophage differentiation induced by phorbol 12-myristate 13-acetate (PMA) in THP-1 cells. The analysis was performed on THP-1 cells which expressed *MLL-AF9* even after PMA-induced monocyte-macrophage differentiation, as previously reported (10). The results indicate that *HOXA4*, *7*, *10*, *11* and *MEIS1* gene expression is potentially involved in the differentiation blockage observed in *MLL-AF9*-induced leukemogenesis.

Materials and methods

The human leukemic THP-1 cell line, carrying the translocation t(9;11)(p22;q23) and expressing the *MLL-AF9* oncogene (11), was cultured and differentiated with phorbol 12-myristate 13-acetate (PMA; 20 nM for 72 h) as previously described (12). For cell viability, cell aliquots were taken daily and counted in a hematocytometer; living cells were evaluated by the

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Table I. Primers employed for RT-PCR.

GenBank Acc. no.	cDNA	Sense primer	Antisense primer	Product size (bp)
NM_002141	HOXA4	ACCCGTTCCCTCCTCCATATAATC	GCAACCAGCACAGACTCTTAACC	200
NM_019102	HOXA5	ACGGCTACGGCTACAATGGC	CCCTCTCTGCTGCTGATGTGG	292
NM_024014	HOXA6	TACACCTCACCTTGTTTCTACC	CGCCTTCGTCATGGAGTG	229
NM_006896	HOXA7	AAATGGGGTTTGGTGTAAATCTG	CCGCTTCTCTGTGAGTTGG	221
NM_152739	HOXA9	TACCACCACCATCACCAC	CACAAGCATAGTCAGTCAGG	232
NM_018951	HOXA10	GCAAAGAGTGGTCGGAAGAAG	CGTCGCCTGGAGATTCATC	254
NM_005523	HOXA11	TGGTCCCTGCTCCTCTAAC	GGCTCAATGGCGTACTCTC	189
NM_002398	MEIS1	AGTGAGCAAGGTGATGGC	CTCGGTTGGACTGGTCTATC	289
NM_004529	MLL-AF9	AGCACTGGTCATCCCGCCTCAG	TCGGCTGCCTCCTCTATTTACAG	382
NM_002046	GAPDH	CAACGGATTTGGTCGTATTG	GGAAGATGGTGATGGGATTT	209

trypan blue dye exclusion test. Differentiation tests, including adherence, phagocytosis, nitro blue tetrazolium (NBT) reduction and CD evaluation, were performed as previously reported (10).

For the reverse transcriptase (RT) reaction, 1 μg of total RNA, isolated with the RNeasy Mini Kit® (Qiagen, Milan, Italy), was pre-treated with RNase-free DNase, heated at 70˚C for 10 min, placed on ice for 1 min, and reverse transcribed by incubation with a mixture containing 0.5 mM dNTP mix, 25 ng/μl oligo(dT)$_{15\text{-}18}$ (Life Technologies Italia, Milan, Italy), 10 mM dithiothreitol, 1X first-strand buffer, 10 units of RNase inhibitor (Amersham Biotech, Milan, Italy), 200 units of SuperScript RT (Life Technologies Italia) and water at a final volume of 20 μl for 1 h at 42˚C. The reaction was stopped by heating at 70˚C for 15 min. The duplex of RNA-DNA was treated with 5 units of RNase H (US Biochemicals, Cleveland, OH) at 37˚C for 20 min, and the amount of single-strand cDNA was evaluated by fluorometry (Victor2 1420 Multilabel Counter, Wallac) with the fluorescent probe Oligreen (Life Technologies Italia) using phage M13+ as a single-strand DNA standard.

For real-time PCR (35 cycles), cDNA was amplified with 2X Platinum® SYBR® Green qPCR SuperMix-UDG (Life Technologies Italia) along with forward and reverse primers (5 pmol each) as listed in Table I. The primer set was designed according to the known sequences reported in GenBank with the help of the Primer 3 program (13). Quantitative PCR was performed in a 36-well Rotor Gene 3000 (Corbett Research, Rotor-Gene™ 3000, version 5.0.60, Mortlake, Australia). Each cycle consisted of a denaturation step at 95˚C for 15 sec, followed by separate annealing (30 sec) and extension (30 sec, 72˚C) steps. Fluorescence was monitored at the end of each extension step. A no-template, no-reverse transcriptase control was included in each experiment. At the end of the amplification cycles, a melting curve analysis was added. Melting temperature analysis for the reaction mix revealed a characteristic melting profile, with a single sharp peak at the typical melting temperature for a given product. The specificity of the product was determined by the generation of a melting curve and by electrophoresis of the amplicons. Samples were run in duplicate, and the average crossing point (CP) value was used for calculations. The analysis of the data was carried out according to the Relative Standard Curve Method (14). The relative quantification value of a target gene, normalized to GAPDH as the internal control gene, is expressed as a number indicating the relative expression compared with that gene.

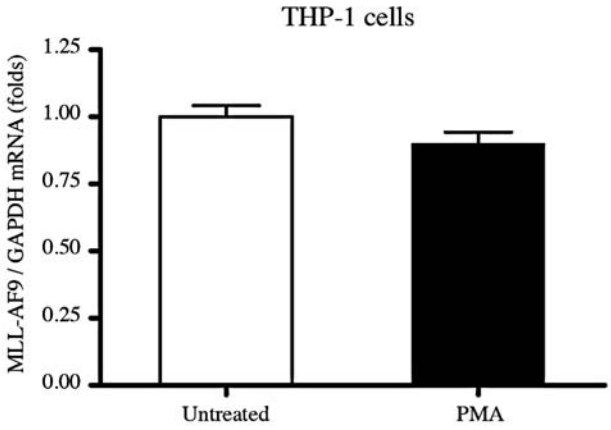

Figure 1. *MLL-AF9* expression in untreated and PMA-treated THP-1 cells. Values represent the means ± SD of two separate experiments.

Results

In order to discriminate between the expression of *MLL-AF9* and the expression of genes induced by *MLL-AF9* expression, we performed all the RT-PCR analyses in THP-1 cells that also expressed *MLL-AF9* after terminal differentiation induced by PMA. Fig. 1 shows no significant difference in *MLL-AF9* expression in untreated THP-1 cells and in those treated with PMA for 72 h. Fig. 2 shows the expression pattern of *HOXA*-code genes (*HOXA4* to *HOXA11*). All these genes were expressed in untreated THP-1 cells. The expression of *HOXA4*, *7*, *10* and *11* genes was down-regulated in THP-1 cells after differentiation induced by PMA. In particular, the strongest down-regulation was observed for *HOXA11* (91% of the control), followed by *HOXA7* and *4* (53 and 52% respectively of the control), and *HOXA10* (45% of the control). *HOXA6* was the only *HOXA* gene whose expression was up-

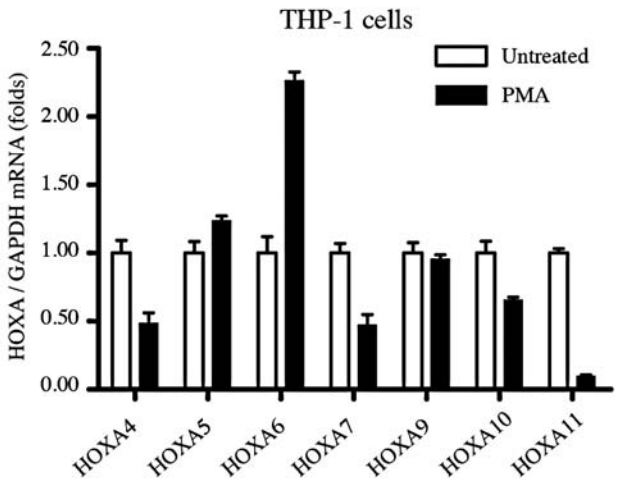

Figure 2. *HOXA*-code expression in untreated and PMA-treated THP-1 cells. Values represent the means ± SD of two separate experiments.

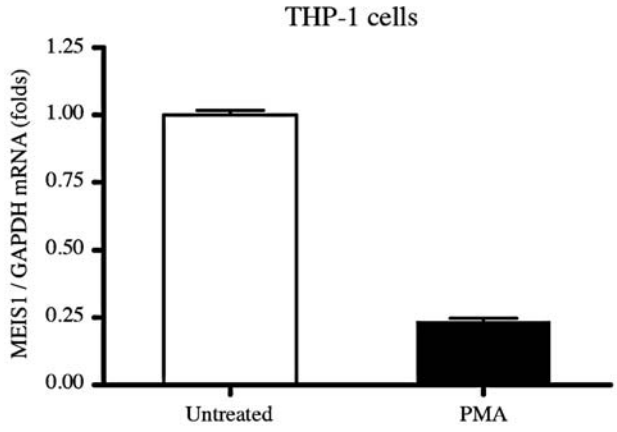

Figure 3. *MEIS1* expression in untreated and PMA-treated THP-1 cells. Values represent the means ± SD of two separate experiments.

regulated (more than twice that of the control). Finally, the difference in the expression of the *HOXA5* and *9* genes was not statistically significant in undifferentiated and differentiated THP-1 cells. To note, the *HOXA8* gene is absent in vertebrates in general. Fig. 3 shows the results of the expression pattern of the *MEIS1* gene, which was expressed in untreated THP-1 cells and down-regulated in differentiated THP-1 cells (72% of control).

Discussion

MLL-AF9 oncogene expression causes leukemic transformation in hematopoietic progenitors. The exact role of *MLL-AF9* is still under investigation. Studies performed in mouse embryonic stem cells indicate that *Mll-AF9* expression causes excessive myeloproliferation (3). Accordingly, we and others reported the inhibition of proliferation in THP-1 cells treated with an MLL-AF9 antisense (8,9). In terms of differentiation blockage, we reported that *MLL-AF9* expression does not affect the terminal differentiation of THP-1 cells (8), and that down-regulation of *MLL-AF9* expression is not obligatory for monocyte-macrophage maturation in AML-M5 cells carrying t(9;11)(p22;q23) (10). These observations suggest that other genes should be examined for differentiation blockage in *MLL-AF9*-related leukemias (8).

It has been shown that, in mouse bone marrow cells, the expression of *Mll-AF9* leads to the overexpression of several genes, including *Hoxa4*, *5*, *6*, *7*, *9*, *10* and *11* (defined as '*Hoxa*-code' genes) and *Meis1*, a Hox cofactor (4-6). *HOX* genes and their cofactors are preferentially expressed in hematopoietic stem cells (HSCs) and immature hematopoietic progenitors, and are down-regulated during terminal differentiation (15). Overexpression of these genes is frequently associated with the leukemogenetic transformation of hematopoietic progenitors. A proposed function of the *HOX*-dependent pathways is the regulation of self-renewal mechanisms in HSCs, and the deregulated expression of the *HOX* genes is crucial for the development of leukemic stem cells (16,17).

In this study, we evaluated the expression patterns of *HOXA*-code genes (*HOXA4* to *HOXA11*) and *MEIS1* during the terminal differentiation of leukemic cells expressing *MLL-AF9*. We performed our analysis using THP-1 cells that also expressed *MLL-AF9* after PMA-induced differentiation in order to discriminate between the expression of *MLL-AF9* and the expression of genes induced by *MLL-AF9* expression (Fig. 1). The results indicate that *HOXA4*, *7*, *10*, *11* and *MEIS1* were down-regulated in THP-1 differentiated cells (Figs. 2 and 3), suggesting these genes play a role in the blockage of terminal differentiation in leukemogenesis induced by *MLL-AF9*. As these down-regulated genes are induced by *MLL-AF9* expression, in future studies, the expression of the MLL-AF9 oncoprotein should be analyzed in differentiated THP-1 cells to evaluate a possible down-regulation of MLL-AF9 at a post-transcriptional level. In regard to *HOXA9* expression, we recorded no significant variation. Nevertheless, it has been reported that HOXA9 protein is phosphorylated by protein kinase C (PKC), and that this phosphorylation is enhanced by PMA, a known inducer of PKC and the inductor of differentiation employed in this study. PKC-mediated phosphorylation of HOXA9 protein impairs its DNA binding ability and induces myeloid differentiation (18). Therefore, the observation that *MEIS1* and most *HOXA*-code genes are down-regulated (transcriptionally or post-transcriptionally for *HOXA9*) during monocyte-macrophage terminal differentiation suggests a role for these genes in the process of differentiation blockage, other than that played in self-renewal processes (17,19,20), in *MLL-AF9*-related leukemias.

To date, little has been reported on the function of *HOXA6*, the only up-regulated *HOXA* gene. In a recent study, *HOXA6* was found hypermethylated, mainly in lymphoid malignancy, suggesting that some *HOX* family members can be frequent targets for gene inactivation and play suppressor roles in leukemia development (21).

In summary, this study supports the theory that, for leukemogenesis induced by *MLL-AF9,* expression of a single *HOXA* gene is not significant. Rather, the expression pattern of several genes in the *HOXA*-code and their cofactors, such as *MEIS1*, is required.

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