

# MLL-ENL Causes a Reversible and *myc*-dependent Block of Myelomonocytic Cell Differentiation<sup>1</sup>

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## ABSTRACT

The translocation t(11;19) is a recurrent feature of a subgroup of acute leukemias occurring in infants. This event fuses the genes *MLL* and *ENL* and creates the leukemogenic oncoprotein MLL-ENL. We studied the effect of retroviral MLL-ENL expression in primary mouse hematopoietic cells and show here that MLL-ENL requires the oncoprotein *Myc* to establish a reversible differentiation arrest of a myelomonocytic precursor population. MLL-ENL-transduced cells proliferated as immature myeloid cells in the presence of interleukin 3. The addition of granulocyte colony-stimulating factor reversed the maturation block set by MLL-ENL and induced the development of mature granulocytes and macrophages accompanied by growth arrest. Gene expression analysis indicated a down-regulation of the proto-oncogene *c-myc* and of several *c-myc* target genes during granulocyte colony-stimulating factor-mediated differentiation. The role of *c-myc* in the MLL-ENL transformation pathway was tested by modulating the effective *Myc* protein concentrations in MLL-ENL transduced cells. Cotransduction of dominant-negative *Myc* neutralized the MLL-ENL effect and precluded transformation. In contrast, constitutive expression of *Myc* cooperated with MLL-ENL and caused the transformation of a cell population with an irreversible maturation arrest.

## INTRODUCTION

The common denominator of the majority of acute leukemias in the early infant age is a chromosomal aberration at the locus 11q23 (for a review see (1, 2). The most frequently observed genetic lesion is a reciprocal translocation that fuses 11q23 to more than 40 known different partner loci distributed over the whole genome (3). Interestingly, translocations involving 11q23 are also prevalent in secondary leukemias that arise after the treatment of primary neoplastic diseases with chemotherapeutic agents that inhibit topoisomerase II (4). Despite this shared genetic feature and contrary to many other translocation-associated leukemia the clinical manifestation of 11q23 translocation-bearing leukemias can be lymphoid, myeloid, or even biphenotypic with markers of both lineages. The latter fact lead to the speculation that the primary target of an 11q23 abnormality is a multipotential hematopoietic precursor cell that gives rise to leukemias in which subsequent myeloid or lymphoid progenitors are blocked in development.

On the molecular level, 11q23 aberrations affect the mixed lineage leukemia gene (*MLL*, also called *HRX* or *ALL-1*). *MLL* codes for a large 3968-amino-acid protein with limited but significant homology to the *Drosophila* epigenetic regulator trithorax (*trx*; 5–8). In the fruit fly, *TRX* is required to maintain, but not to initiate, the expression pattern of the homeobox group genes that control the body plan (9). *TRX* is believed to act by establishing an open state of chromatin that enables transcription of its target genes. Knockout studies in mice

suggest a similar function also for *MLL* (10–12). *In vitro* assays studying the hematopoietic capability of either *MLL*<sup>-/-</sup> embryonic stem cells or *MLL*<sup>-/-</sup> fetal liver cells demonstrated an essential role for *MLL* in hematopoiesis (13, 14). All cloned 11q23 translocations consistently combine the 5' portion of the *MLL* gene in frame with the corresponding fusion partners. The translation of the respective chimeric RNAs yields fusion proteins with novel combinations of functional domains. *MLL* contributes two DNA binding motifs that recognize DNA without strict sequence specificity: the AT-hooks and the CXXC or methyltransferase domain. The role of the respective fusion partners is less clear. There is no apparent unifying feature among the 21 fusion partners cloned to date, except that several of them are either directly or indirectly involved in transcriptional activation. The three most frequent fusion partners AF4, AF9, and ENL [t(4;11), t(9;11), t(11;19)] contain transactivator domains (15, 16) and, consequently, it has been shown that the fusion protein MLL-ENL is a pleiotropic transcriptional transactivator when tested in transient reporter gene assays (17).

Despite substantial sequence information about *MLL* and its fusion partners, the molecular mechanisms of hematopoietic cell transformation by *MLL* fusion proteins remain elusive. Two studies demonstrate the fundamental capability of the translocation products MLL-ENL [t(11;19)] and MLL-AF9 [t(9;11)] to act as leukemogenic proto-oncogenes. Retroviral transduction of primary mouse hematopoietic precursor cells with a virus expressing MLL-ENL immortalized myelomonocytic precursors. These cells were able to elicit a myeloid leukemia on reintroduction into mice. However, MLL-ENL-induced leukemias were only observed after a prolonged latency period of ~3 months (18). In a second study, a translocation t(9;11) was reconstructed at the chromosomal level by a “knock-in” strategy. Modified ES cells were used to generate chimeric mice and the animals were followed in their hematopoietic development. Although an expanded myeloid cell population derived from cells carrying the knock-in allele was detectable shortly after birth, these mice finally succumbed to mostly myeloid leukemias after a latency period of more than 4 months (19, 20). Surprisingly, a fusion of *MLL* with the bacterial *lacZ* gene was able to cause similar myeloid leukemias in this system. Notably, in the case of MLL-*lacZ* expression, the elapsed latency period before the appearance of overt leukemias was approximately twice as long as that observed after expression of the authentic fusion protein MLL-AF9 (21). The use of a model with additional uncontrolled genetic alterations accumulating during the life span of the animals makes an investigation of the progression toward full leukemia difficult. Therefore, the immediate consequence of *MLL* fusion protein expression on cell physiology as well as the nature of potentially cooperating oncogenes is unclear.

In this study, we used a retroviral transduction approach to investigate the direct effects of MLL-ENL on the development of primary hematopoietic cells. We show here that a block in myelomonocytic development caused by MLL-ENL is dependent on the proto-oncogene *c-myc*, and that it is readily reversible. Hematopoietic progenitors transformed by MLL-ENL are growth factor dependent and apoptosis competent, and a normal genetic program of cell differen-

Received 2/13/01; accepted 6/28/01.

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<sup>1</sup>Supported by grants from the Deutsche Forschungsgemeinschaft (SFB466-C7, SFB473-B10, SL27/4-1) and a Ria Freifrau-von-Fritsch Stiftung career development award.

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tiation can be induced by G-CSF.<sup>3</sup> A suppression of Myc activity precludes transformation by MLL-ENL, whereas an ectopic expression of Myc cooperates with the fusion protein. The consequences of these findings for the etiology of leukemias with MLL rearrangements are discussed.

## MATERIALS AND METHODS

**Cell Culture, Media, Growth Factors.** The ecotrophic packaging cell line Phoenix was obtained from Gary Nolan (Stanford, CA). The maintenance of Phoenix cells is outlined on the world wide web.<sup>4</sup> Mouse recombinant growth factors (Strathmann Biotech, Hannover, Germany) were added at the following standard concentrations: IL-3, IL-6, G-CSF, GM-CSF at 10 ng/ml medium; SCF at 100 ng/ml medium. Drug selection was performed in 1 mg/ml G418 (Life Technologies, Inc.) and/or 0.5  $\mu$ g/ml puromycin (Calbiochem, CA). MethoCult (M3234) medium was from Stem Cell Technologies (Vancouver, BC, Canada).

**DNA Manipulation and Plasmid Generation.** Genomic DNA was purified according to standard methods (22). For retroviral transductions, all cDNAs were inserted into either the retroviral vector pMSCV-neo or pMSCV-pac (23). The cDNAs of the human *c-myc* gene and the dominant-negative *c-myc* mutant lacking the amino acids 40–178 of the Myc transactivation domain ( $\Delta$ Myc) were a gift from D. Eick (24, 25).

**RT-PCR, and cDNA Arrays.** Polyadenylated messenger RNA for RT-PCR was purified by oligo d(T) chromatography on latex beads (Oligotex; Qiagen Inc) according to the manufacturer's instructions. Primers used for RT-PCR of MLL-ENL, Myc, and  $\Delta$ Myc were: MLL forward (gcaaacagaaaaagtggctccccc) and ENL reverse (accatccagctgtgagtgaaacct); myc forward (cctagtctgcatgaggggacac) and myc reverse (gcaccagatgttcgaagctgttc); and  $\Delta$ Myc forward (ctcgatctcccttatccagcc) and  $\Delta$ Myc reverse (cctcatctcttcttctctcctcag). The cDNA array analysis was done with the ATLAS mouse cDNA expression array (version 1) from Clontech. The evaluation of the hybridization results was performed with the Clontech Atlas Image analyzer software.

**Virus Production and Retroviral Transduction of Mouse Primary Hematopoietic Cells.** High titer retrovirus supernatants were produced by transient transfection of the packaging cell line Phoenix-E by a standard calcium-phosphate precipitation method (22). The viral titers were in the range of  $1 \times 10^6$  to  $>1 \times 10^7$  colony-forming units/ml depending on the size of the insert in the retroviral vector. The retroviral transduction of primary hematopoietic cells was done according to the method of Lavau *et al.* (18). An overview of the procedure is given in Fig. 5A. BMCs, enriched in noncycling hematopoietic precursors, were recovered from 5-FU-primed Balb/C mice. Previous to infection, the BMCs were activated by a cytokine mixture (IL-3, IL-6, and SCF). After infection by spinoculation, the infected cells were returned to activation medium as above. After overnight activation, duplicates of  $1 \times 10^4$  infected cells were plated under the appropriate drug selection in 0.5 ml each MethoCult murine methylcellulose medium with the addition of SCF, IL-3, IL-6, and GM-CSF. For double transductions, equal volumes of different virus supernatants were mixed before infection, and the infected BMCs were selected simultaneously with G418 and puromycin. Colonies in one of the duplicate wells were stained by the addition of 50  $\mu$ l of 1 mg/ml *p*-iodonitrotetrazolium violet in PBS (INT; Sigma Chemical Co.). INT is converted by living cells to a brown-violet insoluble tetrazolium salt. The colonies in the remaining well were resuspended in medium, counted, and replated as above.

**MTT Assays, and TUNEL Staining.** Cell proliferation was measured either by direct counting of trypan blue-negative cells or by conversion of the tetrazolium dye MTT to a dark violet formazan salt. Apoptosis-mediated DNA fragmentation was measured by the addition of fluorescein-labeled dideoxyuridine onto free DNA ends by and subsequent FACS analysis (TUNEL assay). These procedures were done according to the instructions of the manufacturer (Roche, Penzberg, Germany).

<sup>3</sup> The abbreviations used are: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; SCF, stem cell factor; RT-PCR, reverse transcription-PCR; BMC, bone marrow cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal dideoxy nucleotidyltransferase nick-end labeling; FACS, fluorescence-activated cell sorter; 5-FU, 5-fluorouracil.

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## RESULTS

**MLL-ENL-transduced Cells Are Blocked in Differentiation, IL-3-dependent, and Apoptosis-sensitive.** To investigate the consequences of MLL-ENL expression in hematopoietic cells primary murine BMCs, enriched in hematopoietic precursors by 5-FU treatment, were transduced with MLL-ENL-expressing retroviruses (see "Materials and Methods" and Fig. 4A). As expected, this led to the outgrowth of a myelomonocytic cell population with a high content of early myeloid precursor cells (Fig. 1A). The presence of unrearranged retroviruses was verified by Southern blot analysis, and multiple integration events could be detected (Fig. 1B). The presence of MLL-ENL RNA was confirmed by RT-PCR (Fig. 1C). Three different, independently transduced cell populations were generated for the subsequent experiments (labeled md11 for murine cells transduced with derivative 11). Because there was no significant difference in the experimental results obtained with each of them, only one representative example is shown. A similar MLL-ENL-transduced cell population has been shown previously to elicit acute leukemias after injection into syngeneic mice (18).

Given the factor-independent growth and the resistance against apoptosis of "11q23"-leukemia-derived cell lines, we first wished to test the growth properties of md11 cells. Confirming their early myeloid nature, md11 cells required IL-3 as the sole growth factor for proliferation and survival. The proliferative response was proportional to the added IL-3 concentrations, and a one-half-maximal proliferation stimulus was reached at a concentration of 0.5 ng/ml IL-3 (Fig. 2A). IL-3 deprivation led to a rapid and efficient cell death within 24 h. Cells kept without IL-3 showed the characteristic signs of programmed cell death, including DNA fragmentation and positive TUNEL staining (Fig. 2, B and C). The cells could be kept continuously in culture for more than 6 months in medium supplemented with saturating amounts of IL-3 without showing any signs of senescence. Normal BMCs, transduced with a control retroviral vector, did not proliferate with IL-3 as growth factor (not shown).

**G-CSF Induces Terminal Differentiation of md11 Cells.** During examination of md11 cells cultivated in IL-3, it was noticed that a small number of terminally differentiated granulocytes and macrophages were present in the cell population. To test the possibility that a potential maturation arrest caused by MLL-ENL might be reversible, IL-3 was replaced by the myeloid differentiation factor G-CSF. G-CSF "neutralized" the MLL-ENL-induced maturation arrest and triggered the terminal differentiation of the culture within 8 days.

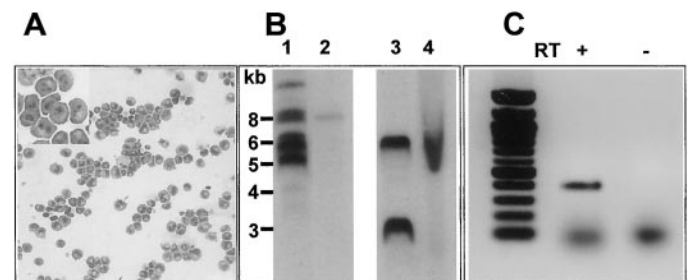


Fig. 1. Characteristics of MLL-ENL-transduced (md11) cells. A, May-Grünwald-Giemsa staining of a cytospin preparation of md11 cells grown in IL-3; large panel,  $\times 20$ ; inset,  $\times 63$ . B, Southern blot analysis. The presence of MLL-ENL virus in the genome of md11 cells was verified by Southern blot. For comparison, the hybridization pattern of the endogenous *MLL* gene was determined in mouse 32D cells. Genomic DNA of md11 cells (Lanes 1 and 3) or of 32D cells (Lanes 2 and 4) was digested with either *Bam*HI (left panel) or *Kpn*I (right panel), blotted, and hybridized with a probe corresponding to the 5' end of the MLL-ENL cDNA. This probe labels one band per integration event in the *Bam*HI blot; two bands indicate unrearranged virus in the *Kpn*I blot. C, RT-PCR analysis. The expression of MLL-ENL RNA in the md11 cells was detected by RT-PCR with primers spanning the breakpoint of the chimeric cDNA.

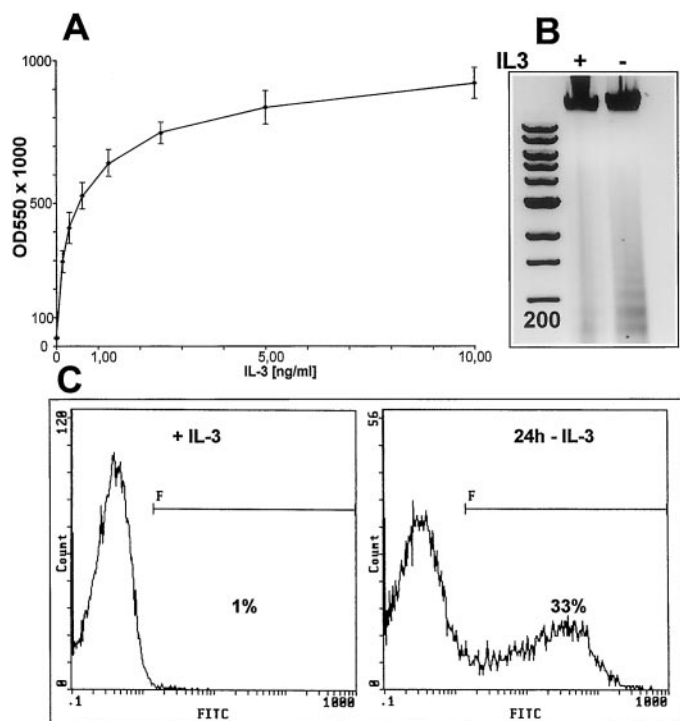


Fig. 2. IL-3 dependency and apoptosis of md11 cells. *A*, growth of md11 cells in response to increasing concentrations of IL-3 measured by the MTT incorporation test. *B*, agarose gel electrophoresis of genomic DNA isolated from md11 cells cultivated as indicated, either in the presence of 10 ng/ml IL-3 or 24 h after the withdrawal of IL-3. A 100-bp ladder, with the lower band corresponding to 200 bp, was used as a size standard. The figure shows an inverted image of the original ethidium bromide-stained gel. *C*, TUNEL staining of md11 cells cultivated with IL-3 or 24 h after IL-3 deprivation.

May-Grünwald-Giemsa staining of cytopsin preparations of md11 cells, cultivated for 6 days in G-CSF, revealed the presence of a high percentage of mature granulocytes. After 8 days, the cultures were dominated by macrophage-like cells, and short-lived granulocytes were mostly extinct (Fig. 3A). G-CSF also initiated a profound proliferation arrest (Fig. 3B). The visible morphological features of differentiation were accompanied by a strong up-regulation of myeloid-specific cell surface markers as detected by FACS staining.

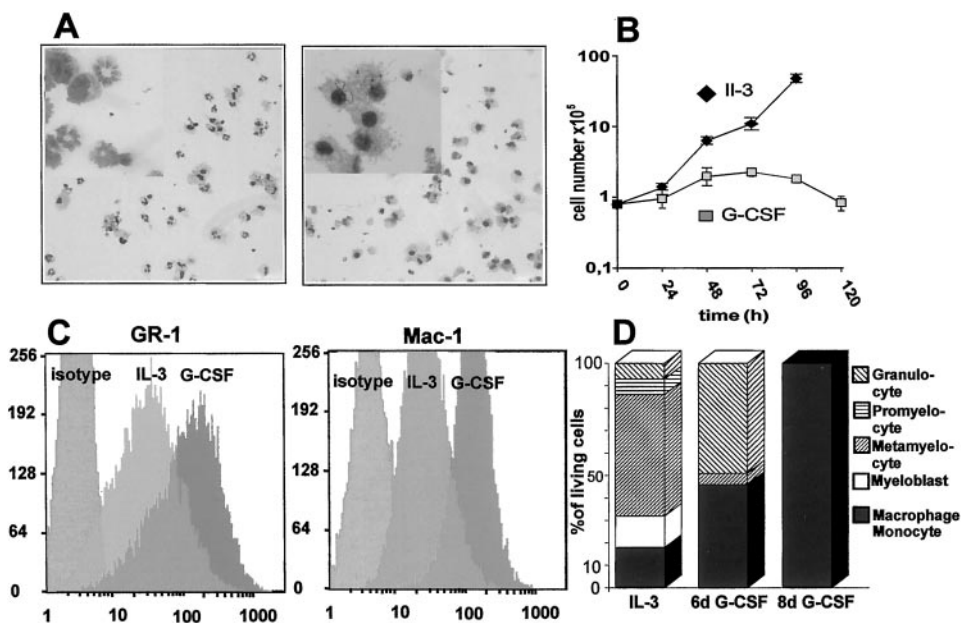


Fig. 3. G-CSF induced terminal differentiation of md11 cells. *A*, May-Grünwald-Giemsa staining of cytopsin preparations of md11 cells after cultivation in G-CSF for 6 days (left panel) or 8 days (right panel); larger panels,  $\times 20$ ; insets,  $\times 63$ . *B*, proliferation of md11 cells in response to 10 ng/ml IL-3 or 10 ng/ml G-CSF. Absolute cell numbers are given. *C*, FACS analysis of cell surface marker expression on cells grown either in IL-3 or for 6 days in G-CSF. The expression of GR-1 (left panel) and Mac-1 (=CD11b, right panel) is shown. *D*, statistical analysis of cell type distribution in the md11 cell population during G-CSF-induced maturation. The cell type assessment was made by morphological criteria on stained cytopsin preparations.

Confirming their primitive myeloid nature, md11 cells that were grown in IL-3 displayed an intermediate surface level of GR-1 and Mac-1 (CD11b). Addition of G-CSF for 6 days elicited a marked shift of both markers toward higher expression levels, which indicated the presence of more mature cells of the myeloid lineage (Fig. 3C). A statistical evaluation of the respective cultures according to morphological criteria supported the view that the MLL-ENL-dependent maturation block was not absolute but could be overcome by the addition of the differentiation factor G-CSF (Fig. 3D). However, occasionally after prolonged culture ( $>4$  months), md11 cells spontaneously generated subclones that lost the differentiation capability and that were able to outgrow the remaining population (not shown).

#### Gene Expression Changes during Differentiation of md11 Cells.

Because G-CSF forced a "reversal" of the MLL-ENL-induced phenotype, we wished to determine whether these morphological changes were also accompanied by a corresponding genetic program. If MLL-ENL initiates a differentiation block by the ectopic activation or repression of target genes, it is possible that a normal expression level of these targets is reestablished after restoration of myeloid differentiation by G-CSF. It seemed, therefore, likely that a subset of the genes that are differentially regulated after G-CSF addition is involved in the maturation arrest that is mediated by MLL-ENL. To identify such genes, a cDNA expression profiling was performed. Polyadenylated RNA was isolated either from md11 cells maintained in IL-3 or from md11 cells 36 h after the addition of G-CSF. Radioactively labeled cDNA was prepared from both RNA preparations using a mixture of 597 gene-specific primers corresponding to the 597 mouse cDNAs immobilized on the Atlas mouse cDNA expression array. Two identical array membranes were hybridized with the respective  $^{32}\text{P}$ -cDNAs, and after a stringent wash procedure, the hybridization signals were recorded with a phosphorimager. The hybridization patterns were compared with each other using the Atlas Image software. This analysis revealed 18 genes with more than a 2.2-fold change in expression after 36 h in G-CSF (Table 1). Two macrophage/monocyte-specific genes coding for a chemokine C-C receptor and for M-CSF (macrophage colony-stimulating factor) were up-regulated in response to G-CSF. The RNA levels of the remaining 16 genes decreased in response to this cytokine. The majority of the genes that were down-regulated after G-CSF treatment coded for growth-



Table 1 Differential gene expression after 36h of G-CSF treatment

Gene	downregulated ratio <sup>a</sup>	upregulated ratio <sup>b</sup>
<i>HSP60</i> , heat shock protein*	3.6	
<i>CD71</i> , transferrin receptor*	3.5	
<i>Nm23-M2</i> , <i>c-myc</i> related transcription factor*	3.0	
<i>mATF4</i> , activating transcription factor4	2.8	
<i>HSP86</i> , heat shock protein	2.7	
<i>YB1</i> , DNA binding protein	2.7	
<i>cyclin A</i>	2.6	
<i>c-myc</i> , proto-oncogene	2.6	
<i>HSP84</i> , heat shock protein	2.4	
<i>DP-1</i> , cell cycle regulatory transcription factor*	2.4	
<i>pim-1</i> , proto-oncogene	2.3	
<i>transcription factor A10</i>	>>	
<i>PCNA</i> , proliferating cell nuclear antigen	>>	
<i>integrin-β</i>	>>	
<i>Rac-1</i>	>>	
<i>ornithine decarboxylase*</i>	>>	
<i>MCP-1RA</i> , monocyte C-C chemokine receptor		2.4
<i>M-CSF</i> , colony stimulating factor 1		2.5

<sup>a</sup> ratio = (expression in IL-3)/(expression in G-CSF), >> expression of the respective gene was not detectable after 36h in G-CSF, \* = Myc target gene.

<sup>b</sup> ratio = (expression in G-CSF)/(expression in IL-3); the ratio cutoff was set to 2.2 fold for all genes investigated.

indicators. Several of these are involved in the cell-cycle regulatory circuit controlled by *c-myc*. Next to the RNA for the proto-oncogene *c-myc* itself, the RNAs of four established *c-myc* target genes (coding for the HSP60 mitochondrial heat-shock protein, the blast marker CD71, ornithine decarboxylase, and the E2F dimerization partner DP-1; Refs. 26, 27) were less abundant in G-CSF-treated cells. In addition, the expression of the proto-oncogene *pim-1*, which is known to cooperate with *c-myc* (28), and of the *myc*-regulatory transcription factor Nm23-M2 (29) also decreased under the influence of G-CSF. The remaining RNAs that were down-regulated after G-CSF treatment corresponded to the following genes: proliferating cell nuclear antigen (*PCNA*), *cyclin A*, the transcription factors *A10* and *ATF4*, the

heat-shock proteins *HSP86* and *HSP84*, *Rac1*, *integrin-β*, and the DNA-binding protein *YB1*.

**MLL-ENL Requires, and Cooperates with, *c-myc* to Block Cellular Differentiation.** The role of *c-myc* in the MLL-ENL-induced transformation process was further examined because this proto-oncogene is of central importance in the regulation of growth versus differentiation during hematopoietic development. For this purpose, BMCs were cotransduced with two different retroviruses expressing MLL-ENL and either a dominant-negative mutant of Myc lacking the transactivation domain ( $\Delta$ Myc) or authentic Myc itself. Cells harboring both viruses were selected by simultaneous addition of G418 and puromycin. The  $\Delta$ Myc protein is capable of forming heterodimers with its normal cellular counterpart Max. The  $\Delta$ Myc/Max heterodimers are still able to bind to E-box-containing promoters but lack the transcriptional transactivator function (24). As controls, BMCs were coinfecting with MLL-ENL/pMSCV-neo, Myc/pMSCV-pac,  $\Delta$ Myc/pMSCV-pac, and with pMSCV-neo/pMSCV-pac (Neo/Puro). The double-transduced cells were tested for their self-renewing capacity, the phenotypic and growth properties, and their differentiation capabilities. In the standard methylcellulose replating assay (Fig. 4A), colonies will form in tertiary rounds of plating only if a cell with self-renewing capabilities has been immortalized. Nontransformed cells will exhaust their proliferative potential and terminally differentiate. In this assay, the coexpression of dominant-negative Myc completely inhibited MLL-ENL-induced colony formation. Apparently, this was not attributable to a simple cell cycle and growth suppression because an equal number of primary MLL-ENL/ $\Delta$ Myc and MLL-ENL/neo colonies were observed (Fig. 4B). In addition, the total cell count of MLL-ENL/ $\Delta$ Myc and MLL-ENL/Neo cells was comparable in the primary round of plating (Fig. 4C). In contrast, cells cotransduced with MLL-ENL and wild-type Myc consistently yielded a higher number of colonies in third-round platings when compared with the MLL-ENL/Neo control cells. In the absence of MLL-ENL, neither cells expressing Myc nor cells expressing  $\Delta$ Myc formed

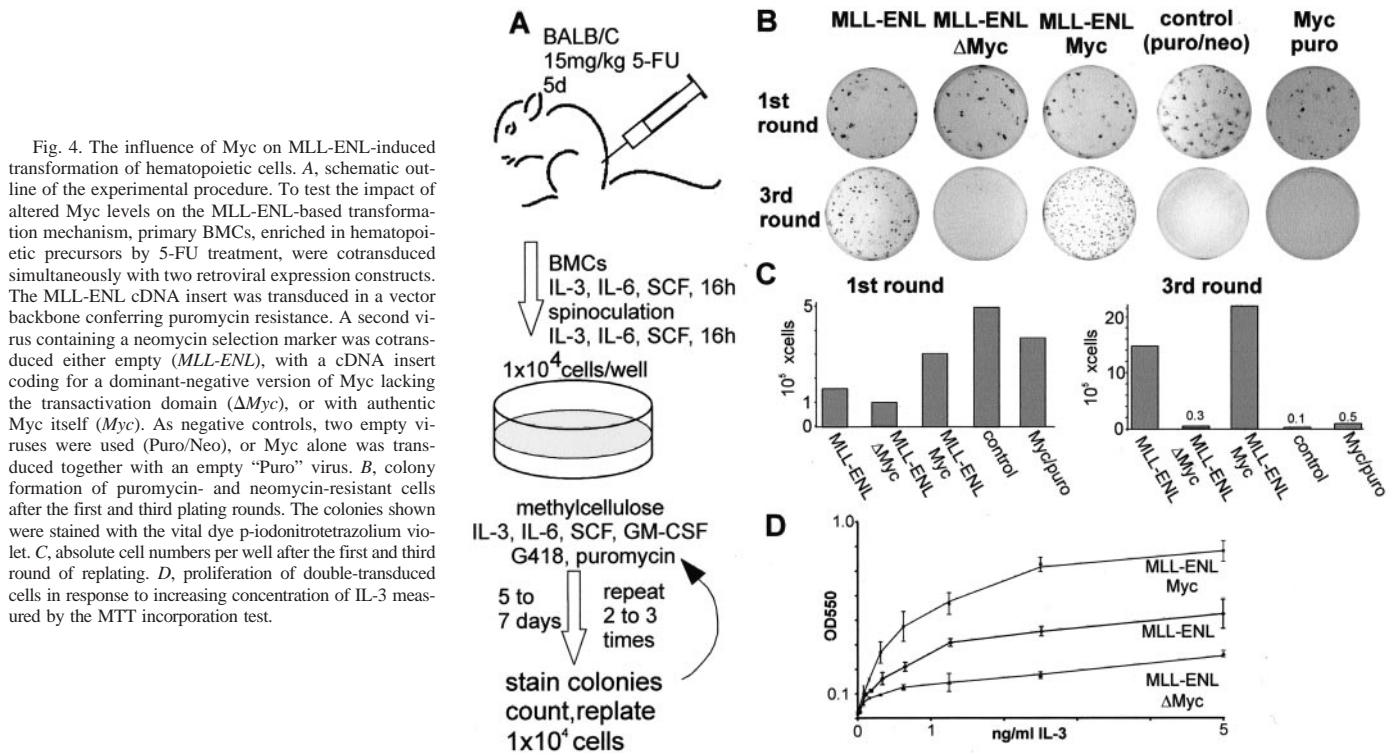
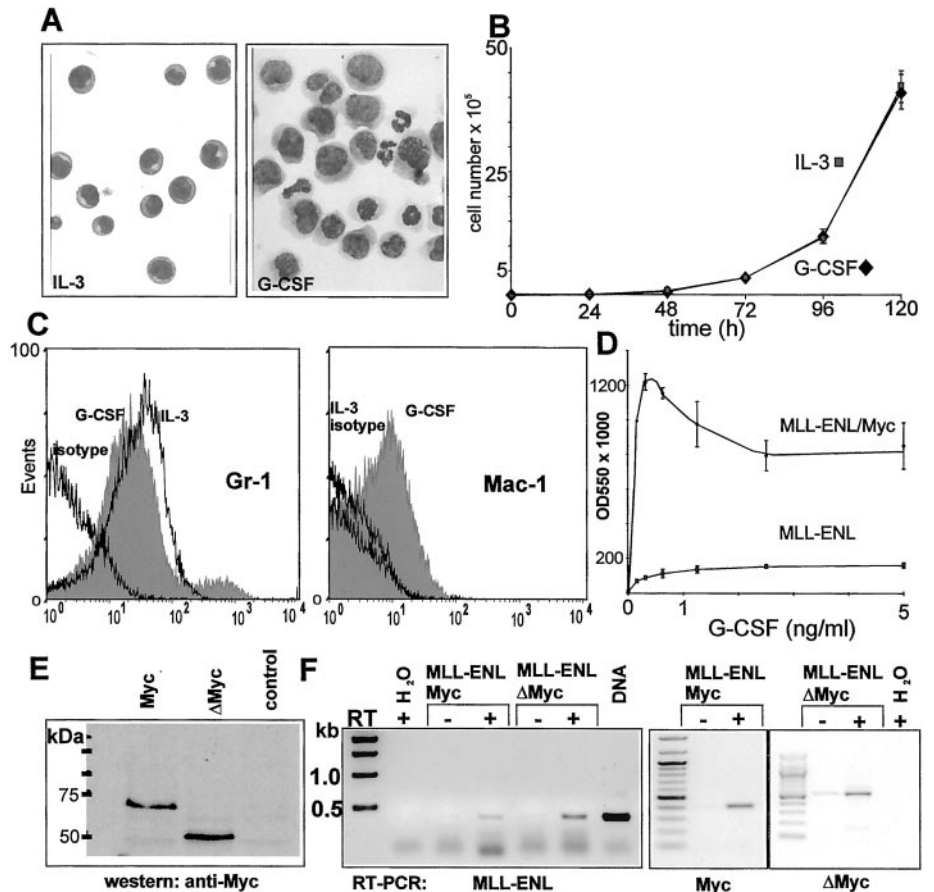


Fig. 4. The influence of Myc on MLL-ENL-induced transformation of hematopoietic cells. **A**, schematic outline of the experimental procedure. To test the impact of altered Myc levels on the MLL-ENL-based transformation mechanism, primary BMCs, enriched in hematopoietic precursors by 5-FU treatment, were cotransduced simultaneously with two retroviral expression constructs. The MLL-ENL cDNA insert was transduced in a vector backbone conferring puromycin resistance. A second virus containing a neomycin selection marker was cotransduced either empty (*MLL-ENL*), with a cDNA insert coding for a dominant-negative version of Myc lacking the transactivation domain ( $\Delta$ Myc), or with authentic Myc itself (*Myc*). As negative controls, two empty viruses were used (Puro/Neo), or Myc alone was transduced together with an empty "Puro" virus. **B**, colony formation of puromycin- and neomycin-resistant cells after the first and third plating rounds. The colonies shown were stained with the vital dye p-iodonitrotetrazolium violet. **C**, absolute cell numbers per well after the first and third round of replating. **D**, proliferation of double-transduced cells in response to increasing concentration of IL-3 measured by the MTT incorporation test.

Fig. 5. Characterization of MLL-ENL/Myc cells. *A*, May-Grünwald-Giemsa staining of cytospin preparations of MLL-ENL/Myc cells after cultivation in IL-3 (*left panel*) or in G-CSF for 6 days (*right panel*);  $\times 63$ . *B*, growth curve of MLL-ENL/Myc cells cultivated in 10 ng/ml IL-3 or 10 ng/ml G-CSF. Absolute cell numbers are given. *C*, FACS analysis of cell surface marker expression on cells grown either in IL-3 (*black outline*) or for 6 days in G-CSF (*shaded curve*). The expression of GR-1 (*left panel*) and Mac-1 (= CD11b; *right panel*) is shown. *D*, proliferation of MLL-ENL/Myc and MLL-ENL cells in response to G-CSF as determined by the MTT incorporation assay. *E*, Western blot analysis of the Myc and  $\Delta$ Myc proteins expressed by the respective retroviruses in the packaging cell line. *Lane 1*, Myc; *Lane 2*,  $\Delta$ Myc; *Lane 3*, empty virus. The blot was probed with a monoclonal  $\alpha$ -Myc antibody; *kDa*, *M<sub>r</sub>* in thousands. *F*, RT-PCR analysis of the expression of MLL-ENL (*left panel*), Myc (*middle panel*), and  $\Delta$ Myc (*right panel*). RNA was isolated and reverse transcribed from MLL-ENL/ $\Delta$ Myc and MLL-ENL/Myc cells. The expected sizes for the amplification products were 380 bp for MLL-ENL, 400 bp for Myc, and 550 bp for the  $\Delta$ Myc RNA. Some residual DNA in the RNA preparation gave rise to weak bands in the control reactions without reverse transcriptase. The standards used are a 1-kb and a 100-bp ladder. *H<sub>2</sub>O*, negative control reaction without template; *DNA*, positive control with MLL-ENL cDNA as template. Reverse transcriptase was added as indicated (*RT*).



colonies in third-round platings (Fig. 4*B* and not shown). Similar results were obtained when the proliferation of the cells in response to IL-3 was measured 3 days after explantation from third-round methylcellulose cultures. MLL-ENL/Myc cells proliferated more vigorously and reached higher cell densities than MLL-ENL cells (Fig. 4*D*). Both cell populations could be grown continuously ( $>4$  weeks) in liquid medium without any signs of decrease in vitality. Cells coexpressing the dominant-negative Myc, however, proliferated only weekly and stopped growth completely after  $\sim 2$  weeks in liquid culture. The Puro/Neo negative control cells did not grow in liquid culture (not shown). Myc also had an effect on the differentiation potential of md11 cells. MLL-ENL/Myc cells remained arrested in an earlier state of myeloid differentiation (Mac-1 negative) and showed less spontaneous differentiation than MLL-ENL cells (Fig. 5*A*). Additionally, G-CSF did not induce growth arrest and differentiation of MLL-ENL/Myc cells but, instead, stimulated a proliferative response similar to that from IL-3 (Fig. 5*B*). G-CSF treated MLL-ENL/Myc cells up-regulated surface Mac-1, but only a small subpopulation showed an increase in GR-1. The generation of a GR-1<sup>+</sup> subpopulation after G-CSF treatment was accompanied by the appearance of a few mature granulocytes in cytospin preparations (Fig. 5, *A* and *C*). The majority of the cell population, however, remained arrested in a premature state and continued to proliferate in the presence of G-CSF. Despite the growth promoting effects of Myc, it did not render the cells growth-factor independent. MLL-ENL/Myc cells responded up to an optimum G-CSF concentration of  $\sim 0.5$  ng/ml with an increase in the proliferation rate and did not grow without this cytokine. Higher than optimal G-CSF concentrations lead to a slight reduction in the proliferation rate (Fig. 5*D*). The integrity of the Myc and  $\Delta$ Myc retroviral inserts was verified by immunoblot analysis of protein

extracts prepared from the viral packaging cells (Fig. 5*E*). The MLL-ENL RNA as well as the Myc and the  $\Delta$ Myc transcript was detected by RT-PCR in MLL-ENL/Myc and MLL-ENL/ $\Delta$ Myc cells (Fig. 5*F*).

## DISCUSSION

Translocations affecting the chromosomal locus 11q23 are the hallmark of a class of aggressive leukemias that are especially prevalent in infants. Much effort has been devoted to prove that the expression of MLL fusion proteins generated by the translocation events constitutes the underlying cause of the respective leukemias. Here, we describe the consequences of the expression of MLL-ENL, the product of a t(11;19), for the development of mouse hematopoietic precursor cells. MLL-ENL sets a reversible block in myelomonocytic differentiation and causes the transformation of factor-dependent, highly apoptosis-sensitive myeloid cells. Furthermore, we show that the proto-oncogene *c-myc* is a mediator of MLL-ENL-dependent transformation. The presence of active Myc is essential for the MLL-ENL-induced differentiation arrest, and excess Myc renders this process irreversible.

The md11 cells described here are in several aspects significantly different from cells that were established from 11q23 leukemias: (*a*) a characteristic clinical feature of this type of leukemia is the frequent treatment failure attributable to the resistance of the leukemic cells to high doses of chemotherapeutic agents (30). Recent reports underscore these observations and describe an unusual resistance of four different patient-derived cell lines against apoptosis (31, 32). md11 cells, in contrast, are highly apoptosis sensitive; (*b*) unlike md11 cells many of the cell lines derived from patients carrying a 11q23 translocation were not dependent on growth factors other than those

contained in the serum of the culture medium; and (c) most of these lines did not show any differentiation capability (33, 34). These differences are supportive of the hypothesis that the expression of an MLL-ENL fusion protein might only be the first genetic event and has to be followed by additional mutations before acute leukemia arises. These secondary mutations would render the cells finally apoptosis-resistant, growth-factor independent, and refractory toward differentiation stimuli. Interestingly, a series of studies that recreated a t(9;11) translocation in a natural setting by a genomic knock-in strategy observed a distinct two-phase etiology of leukemia (19, 20). Dobson *et al.* (20) detected an elevated level of myeloid cells in the blood of the AF9 knock-in mice shortly after birth but no clinical signs of leukemia were present yet. After up to 6 months of latency, the animals finally succumbed to acute leukemias. This would be consistent with the initial presence of a population of myeloid cells with a partial block in differentiation similar to md11 cells. A subsequent genetic event or events might convert this initial myeloproliferative disease into an acute leukemia. The multistep nature of leukemias with an 11q23 translocation is also corroborated by the discovery of three genes that have been found mutated in leukemia cells that are isolated from patients. Dominant-negative mutants of the transcription factor *Ikaros* and an abrogation of *INK4A* expression by promoter methylation were frequent in these leukemias (35, 36). In addition, in rare cases, activated *ras* mutants have been detected (37).

The experiments presented here suggest that the proto-oncogene *c-myc* is not only a likely candidate for a secondary mutation but it seems to be also intricately involved in the biological function of MLL-ENL. The nuclear phosphoprotein c-Myc controls cell cycle progression and has a central role in growth regulation. Consequently, it is frequently found as an activated oncogene in human neoplastic disease (38). The expression of *c-myc* is normally tightly controlled. Forced *myc* expression can drive quiescent cells into cycle and is able to block differentiation. When coexpressed with an oncogenic partner such as *ras*, *c-myc* can induce transformation of primary cells. A plausible model to explain the effects of Myc on the MLL-ENL-induced transformation would postulate that MLL-ENL cooperates with Myc in a common pathway to activate the transcription of an overlapping set of target genes. Alternatively, MLL-ENL and Myc might act in parallel pathways that converge on essential targets that cannot be sufficiently activated by either MLL-ENL or Myc alone. The intricate connection of MLL fusion proteins and Myc was corroborated in a recent study of Galoian *et al.* (39). In these experiments, not only MLL-ENL but also three other MLL fusion proteins functioned as specific transactivators for promoters that contained myc-type E-boxes. Most importantly, this transactivator property of MLL-ENL was essentially dependent on the presence of the Myc protein itself. The MLL fusion proteins did not transactivate E-box promoters in Myc<sup>-/-</sup> cells. Because the levels of Myc protein were unaltered in cells expressing MLL fusion proteins, the cooperation apparently augments the intrinsic ability of Myc to activate certain target promoters. It is easy to imagine how a moderate increase in the concentration of Myc targets might lead to a reversible differentiation arrest that can be overcome by a strong maturation stimulus, *e.g.*, by G-CSF. An additional overexpression of the Myc protein might amplify the cooperative effect with MLL-ENL and finally drive the cells beyond a threshold level that makes the maturation block irreversible. Although the activation of Myc targets seems to be necessary for the MLL-ENL transformation mechanism, it is not sufficient. An overexpression of Myc alone did not lead to transformation of the hematopoietic cells and, therefore, MLL-ENL must have additional effects that are required to elicit a true transformation. The availability of the bone marrow transduction system will allow us to dissect additional

molecular details of the leukemogenesis caused by MLL-ENL and to identify other potentially cooperating oncogenes.

## ACKNOWLEDGMENTS

We thank Dirk Eick (Gesellschaft für Strahlen- und Umweltforschung GSF, Munich, Germany) for the kind gift of the *c-myc* cDNAs and Gary Nolan (Stanford University, Stanford, CA) for the phoenix packaging cell line. We are grateful for technical advice by Jorge DiMartino and for continual support from Georg Fey.

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