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Myc Inhibits p27-Induced Erythroid Differentiation of Leukemia Cells by Repressing Erythroid Master Genes without Reversing p27-Mediated Cell Cycle Arrest†‡

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Inhibition of differentiation has been proposed as an important mechanism for Myc-induced tumorigenesis, but the mechanisms involved are unclear. We have established a genetically defined differentiation model in human leukemia K562 cells by conditional expression of the cyclin-dependent kinase (Cdk) inhibitor p27 (inducible by Zn2+) and Myc (activatable by 4-hydroxy-tamoxifen). Induction of p27 resulted in erythroid differentiation, accompanied by Cdk inhibition and G0 arrest. Interestingly, activation of Myc inhibited p27-mediated erythroid differentiation without affecting p27-mediated proliferation arrest. Microarray-based gene expression indicated that, in the presence of p27, Myc blocked the upregulation of several erythroid-cell-specific genes, including NFE2, JUNB, and GATA1 (transcription factors with a pivotal role in erythropoiesis). Moreover, Myc also blocked the upregulation of Mad1, a transcriptional antagonist of Myc that is able to induce erythroid differentiation. Cotransfection experiments demonstrated that Myc-mediated inhibition of differentiation is partly dependent on the repression of Mad1 and GATA1. In conclusion, this model demonstrates that Myc-mediated inhibition of differentiation depends on the regulation of a specific gene program, whereas it is independent of p27-mediated cell cycle arrest. Our results support the hypothesis that differentiation inhibition is an important Myc tumorigenic mechanism that is independent of cell proliferation.

c-Myc (Myc herein after) is an oncogenic transcription factor of the helix-loop-helix/leucine zipper (HLH-LZ) protein family that elicits a variety of biological responses related to cell cycle control, genomic instability, immortalization, energetic metabolism, ribosome biogenesis, apoptosis, intercellular communication, and control of cell differentiation (for reviews, see references 5, 17, 21, and 40). Myc forms heterodimers with the protein Max and the Myc-Max dimers bind to E-boxes in regulatory regions to transactivate genes. Also, an important number of Myc target genes (30 to 50% across different studies) are repressed by Myc-Max in an E-box-independent manner (18, 27, 39, 58; see also the Myc target gene database [www.myccancergene.org]). The mechanism for Myc-mediated transactivation involves chromatin acetylation, whereas the mechanisms for Myc-mediated transrepression remain poorly defined, with the exception of a few genes (1, 9, 11, 41). On the other hand, Max form dimers with proteins of the Mad family. Mad proteins are also HLH-LZ proteins that function as Myc antagonists, since Mad-Max dimers repress transcription upon binding to E-boxes (5, 21). Consistent with the Myc effects on cultured cells and transgenic models, deregulated expression of Myc is found in a wide array of human cancers, in many cases associated with disease progression (30, 36).

Work in different mouse models has demonstrated that Myc promotes differentiation in some tissues by expanding the stem cell population (53, 56) or by promoting both proliferation and differentiation of immature precursors (22). However, enforced Myc expression blocks differentiation of a wide variety of cell types both in vitro and in vivo (for reviews, see references 21 and 40). Actually, inhibition of differentiation was one of the first biological effects described for Myc (8, 16, 44). However, in contrast to the extensive research carried out on the mechanisms by which Myc enhances proliferation, the mechanisms for the Myc-mediated suppression of differentiation are much less known. Since proliferation and differentiation are usually mutually exclusive and Myc drives cells into proliferation, it has been argued that Myc prevents terminal differentiation by blocking the cell cycle exit (21, 40). Consistently, Myc induces the expression of genes that promote cell cycle progression (cyclins D2 and E1, Cdk4) and represses cell cycle inhibitors as p21\(^{WAF1}\) and p27\(^{KIP1}\) (referred to as p27 hereafter) (30).

p27 was originally described as a negative regulator of cell cycle progression through the inhibition of cyclin-dependent kinases (Cdks) (37, 46). However, p27 has also been involved in biological functions unrelated to cell cycle, including the differentiation of erythroid precursors (14, 48). Regarding he-
matopoisis, p27 is expressed in CD34+ progenitor cells and in the primitive erythroid precursors (48, 55), but p27-deficient mice do not show gross abnormalities in the hematopoietic lineages (reviewed in reference 34).

A functional antagonism between Myc and p27 in proliferation has been well established: Myc and p27 loss cooperates in animal carcinogenesis models (31), and several reports demonstrate the ability of Myc to abrogate p27 function (7, 43, 51, 54) and expression (54). However, in sharp contrast to the information on the antagonism between Myc and p27 in proliferation, the possible Myc-p27 cross talk in differentiation has not been investigated.

The study of Myc effects on differentiation has been impaired by the complex array of pathways activated by cytokines and chemicals used as differentiation inducers in most model systems. We previously showed that induction of p27 results in erythroid differentiation of K562 (35), and here we have investigated the role of Myc in this genetically defined differentiation model. We found that Myc blocks p27-mediated differentiation but that it cannot rescue the p27-dependent proliferation arrest and Cdk inhibition. We also report that Myc blocks the upregulation of Mad1 and of a set of erythroid-differentiation-determining genes such as the transcription factor GATA1.

MATERIALS AND METHODS

Cell culture, differentiation assays, and transfections. K562 cells were grown in RPMI 1640 medium supplemented with 8% fetal calf serum and antibiotics. Kp27MER and KMER4 sublines were generated electroporating the pBABE-MycER plasmid (28) into Kp27-5 and KMT sublines, which have been described previously (35). The Kp27pBP and KMTpBP sublines were generated by electroporating Kp27-5 and KMT with pBABE-puro vector. A total of 10⁴ cells were electroporated with 20 µg of DNA (200 V and 1 mF in a Bio-Rad gene pulser) and selected with 1 µg of puromycin (Sigma)/ml. Cells (2.5 × 10⁵ cells/ml) were treated with ZnSO₄ to induce p27 expression, and 4-hydroxy-tamoxifen (4HT; Sigma) to activate MycER. Unless otherwise stated, ZnSO₄ and 4HT were used in culture media (control, ZnSO₄, 4HT, and ZnSO₄ + 4HT) in Kp27MER and K562. The interaction network for differentially expressed genes was generated by using Ingenuity Pathways Analysis software.

Immunofluorescence and immunoblotting. For immunofluorescence, Kp27MER cells were treated with ZnSO₄ and/or 4HT for 12 and 24 h and immunostained with the anti-Myc and anti-p27 antibodies, as well as with DAPI (4’,6-diamidino-2-phenylindole) to stain nuclei. A total of 2 × 10⁵ to 5 × 10⁷ Kp27MER cells were centrifuged at 1,500 rpm for 5 min, resuspended in 8 µl of PBS, and dried on microslides. Cells were fixed for 15 min in 3.7% paraformaldehyde in PBS at room temperature and permeabilized with 0.5% Triton X-100 for 30 min. The cells were then incubated with primary (16 h at 4°C), washed, and incubated with secondary antibodies (1 h at room temperature). The primary antibodies used were anti-Myc rabbit polyclonal antibody and anti-p27 mouse monoclonal antibody (both diluted 1:25). Secondary antibodies were conjugated with Texas Red or FITC (Jackson Laboratories). Cells were mounted with antifading mounting medium Vectashield (Vector Laboratories) with DAPI to visualize the nuclei. Images were recorded by using a Bio-Rad MRC 1024 confocal laser microscope. Immunoblots were performed as previously described (35). Blots were revealed with an ECL system (Amersham). The antibodies used are described in Table S2 in the supplemental material.

Immunoprecipitations and kinase activity assays. Protein extracts (500 µg per assay) were immunoprecipitated with 1 µg of anti-cyclin D2, cyclin D3, and Cdk2 and p27 antibodies (see Table S2 in the supplemental material). Immunoprecipitations and kinase activity assays were performed as described previously (35).

Microarray data were deposited in the ArrayExpress database under accession number E-MEXP-1772.

RESULTS

Generation of K562 sublines with conditional expression of p27 and Myc. We previously reported that induction of p27 in K562 human myeloid cells resulted in G₁ arrest and erythroid differentiation (35). We took advantage of this differentiation system to ask whether Myc interferes with p27-mediated differentiation and to study the possible mechanisms involved. For this purpose, we first generated cells with conditional expression of both p27 and Myc. Kp27-5 cells (which carry a Zn²⁺-inducible p27 allele) were stably transfected with an expression vector for the fusion protein MycER, where Myc activity is activated by 4HT (28). One of the transfectants, termed Kp27MER, was selected, and immunoblot analysis confirmed that the cells expressed MycER and retained p27 induction by Zn²⁺. The activation of MycER by 4HT was assessed, first, by the Myc downregulation of endogenous Myc (Fig. la), an effect observed in many cell lines, including K562 (12, 42). We further confirmed the activation of MycER by transactivation assays with a luciferase reporter carrying E-boxes (Fig. 1B). Immunofluorescence studies demonstrated that, in Kp27MER cells, ZnSO₄ induced a dramatic accumulation of p27 in cell nucleus and that the concomitant activation of Myc by 4HT did not modify this p27 localization (Fig. 1C). Together, the results demonstrated the activation of MycER in response to 4HT in Kp27MER cells.

Myc inhibits p27-induced erythroid differentiation. We next sought to determine whether Myc could antagonize the ery-
thyroid differentiation mediated by p27. Kp27MER cells were treated with ZnSO₄ to induce p27 and 4HT to activate Myc, and the erythroid differentiation was analyzed first by the fraction of cells containing hemoglobin as determined by the benzidine test. This fraction was reduced by 4HT in Kp27MER cells but not in control Kp27pBP cells (Fig. 2A). Myc effect was further confirmed by the expression of erythroid-cell-specific genes as glycophorin A (GYPA), erythropoietin receptor (EPOR), ζ-globin, and ε-globin. mRNA levels of these genes were induced by p27, and this induction was blocked by 4HT (Fig. 2B). Finally, Myc also reversed the morphological differentiation into erythroid phenotype induced by p27, as shown by the dramatic reduction in the number of basophilic erythroblast-like cells (Fig. 2C). Altogether, the results show that Myc inhibited p27-induced differentiation.

**Myc does not antagonize the proliferation arrest mediated by p27.** As proposed for other models, we explored the possibility that Myc could inhibit erythroid differentiation by maintaining the cells in a proliferative state. When p27 was induced with 75 μM Zn²⁺, Kp27MER cells underwent a rapid growth arrest, similar to that induced by p27 in control cells (Kp27pBP) (Fig. 3A). In agreement with the growth determinations, p27 induction was accompanied by S-phase depletion and G₁-phase arrest (Fig. 3B). Surprisingly, the activation of Myc by 4HT in these conditions did not reverse this arrest (Fig. 3A), and cell cycle analysis revealed the G₁-phase arrest provoked by p27 upon treatment with 75 μM Zn²⁺ was not modified by Myc (i.e., by 4HT treatment) (Fig. 3B). Treatment with lower Zn²⁺ concentrations (50 μM) resulted in moderate p27 levels (Fig. 3C) and a less severe proliferation arrest than with higher p27 levels (i.e., with 75 μM Zn²⁺) (Fig. 3A). Consistently, moderate p27 levels (i.e., with 50 μM Zn²⁺) induced a weaker differentiation, which was also blunted by Myc (not shown). In the presence of moderate p27 levels, however, Myc provoked a slight but consistent increase in proliferation (Fig. 3A). This effect depended on Myc since 4HT did not modify the proliferation rate of control Kp27pBP cells (Fig. 3A). Consistently, Myc provoked an increase in the fraction of cells in S phase when the cell cycle was analyzed (Fig. 3B). In agreement with this result, Myc reversed the moderate repression of the mitotic cyclin B1 in cells treated with 50 μM Zn²⁺, as determined by immunoblot analysis (Fig. 3D). In contrast, the ex-
expression of the erythroid gene \( \gamma \)-globin was similarly blunted by Myc in the presence of 75 or 50 \( \mu \)M Zn\( \text{SO}_4 \). The results were confirmed by measurements of DNA synthesis by BrdU incorporation assays. As expected, DNA synthesis was halted in Kp27MER cells treated with 75 or 50 \( \mu \)M Zn\( \text{SO}_4 \) (i.e., high \( \gamma \)-globin levels). Activation of Myc did not relieve this inhibition (Fig. 3E). However, DNA synthesis was not totally inhibited in cells treated with 50 \( \mu \)M Zn\( \text{SO}_4 \) (moderate \( \gamma \)-globin levels), and Myc induced a moderate increase in DNA synthesis (Fig. 3E). We conclude that Myc inhibits \( \gamma \)-globin-induced erythroid differentiation without reversing the \( \gamma \)-globin-mediated cell cycle arrest.

Myc does not reverse \( \gamma \)-globin-mediated inhibition of Cdkks. The best-defined biochemical activity of \( \gamma \)-globin is the inhibition of Cdk activity. Thus, we sought to determine whether Myc impaired this activity in the K562 model in conditions where Myc is blocking differentiation. We first compared the expression of cyclins D and Cdk4, Cdk6, and Cdk2 in cells arrested and differentiated by \( \gamma \)-globin (i.e., treated with Zn\( \text{SO}_4 \)) and in cells with the differentiation blocked after Myc activation (i.e., treated with Zn\( \text{SO}_4 \) and 4HT) (Fig. 4A). \( \gamma \)-globin induced the accumulation of cyclins D, an effect likely due to protein stabilization since there was no upregulation of cyclins D mRNA (data not shown). Myc also induced a small increase in cyclin A, cyclin D2, Cdk4, and Cdk2. No significant changes were observed in parental K562 cells treated with Zn\( \text{SO}_4 \) or 4HT (results not shown).

In most models, the G1-phase arrest by \( \gamma \)-globin is associated with retinoblastoma (RB) hypophosphorylation. Immunoblot experiments show that this was also the case in Kp27MER treated with Zn\( \text{SO}_4 \). Interestingly, in the presence of \( \gamma \)-globin, most of the RB remained hypophosphorylated upon 4HT addition (Fig. 4B). We next analyzed the levels of RB phosphorylated in Ser780 and Thr821 using phospho-specific antibodies. Phosphorylation in Ser780 and Thr821 have been reported as specific for Cdk4/6 and Cdk2, respectively (25, 57). Immunoblot results confirmed that \( \gamma \)-globin profoundly repressed RB phosphorylation and that Myc could reverse none of them.

We next studied the kinase activity in cyclin D2 and cyclin D3 complexes, the most prevalent cyclin D forms in K562 (Fig. 4A and data not shown) using RB protein as substrate. In this setting, the kinase activity assayed is mostly due to Cdk2, Cdk4, and Cdk6. The kinase activity was dramatically inhibited by \( \gamma \)-globin, and Myc only slightly reversed this inhibition (Fig. 4C), which is consistent with the small increase in phospho-RB observed in 4HT-treated cells (Fig. 4B). K562 cells are deficient in p15^{INK4b} and p16^{INK4a} (13), and thus these proteins cannot contribute to Cdk4/6 inhibition in this system. Since RB is also the substrate for Cdk2 kinase activity, we assayed the kinase activity after immunoprecipitation with anti-Cdk2 antibody and in the presence or absence of Myc (i.e., with or without 4HT treatment). The results showed a dramatic inhibition of Cdk2 activity in \( \gamma \)-globin-expressing cells, which was unchanged by Myc (Fig. 4C). Thus, Myc cannot reverse the inhibition of Cdk4/6 and Cdk2 elicited by \( \gamma \)-globin.
Cdk2, Cdk4, and Cdk6 were found in p27 immunoprecipitates, and Myc did not modify their levels (Fig. 4D). However, Myc activation also resulted in greater amounts of cyclins D. Thus, we also tested the possibility that Myc modified the affinity of p27 for cyclin D-Cdk4/6 complexes. However, Myc activation did not change the relative amounts of p27, Cdk4, and Cdk6 present in cyclin D2 and D3 immunoprecipitates (Fig. 4E) and correlated to the amounts present in total extracts (Fig. 4A and data not shown). The results are again consistent with the lack of recovery in Cdk activity (Fig. 4C). Taken together, the data demonstrate that Myc inhibited p27-induced erythroid differentiation without reversing the p27-dependent inhibition of Cdks, a result that is consistent with the inability of Myc to reverse p27-mediated proliferation arrest.

**Myc antagonizes the upregulation of erythroid-cell-specific genes mediated by p27.** Since Myc inhibited Kp27MER differentiation without rescuing the cells from the G1 arrest imposed by p27, this model provided an ideal opportunity to identify proliferation-independent targets of Myc that may explain its activity as a differentiation inhibitor. We therefore carried out microarray analysis with the Affymetrix platform testing RNA from Kp27MER cells either untreated or treated for 12 h with ZnSO4, 4HT, or both. The experimental conditions and associated phenotypes, as well as the number of regulated genes, are schematized in Fig. 5A. We focused in the genes regulated by Myc in the presence of p27, comparing the transcriptomes of Kp27MER treated with ZnSO4 with cells treated with ZnSO4 plus 4HT. After subtracting the genes changed in control samples, filtering, and statistical analysis, we found that Myc regulated 200 genes with ≥2.3-fold change, being 121 genes downregulated (see Table S3 in the supplemental material). The clustering analysis of these genes showed that all Myc-expressing cells cluster together (Fig. 5B). This data set was further analyzed with the Ingenuity Pathways software to reveal the network of interactions between differentially regulated genes in the Kp27MER cells after induction of Myc and thus assaying the possible relevance of Myc activation in this model. The results revealed that the top-ranked network had Myc at the most significant node of interactions among proteins whose expression levels (at the mRNA level) changed between cells treated with ZnSO4 and cells treated with ZnSO4 plus 4HT (Fig. 5C). This result argues that Myc is responsible for the phenotypic change of Kp27MER cells upon addition of 4HT.

**Myc downregulates erythroid genes.** Microarray analysis revealed that a significant fraction of the genes downregulated by Myc in the presence of p27 were erythroid cell related (see Table S4 in the supplemental material), and the same result was observed for other erythroid genes (e.g., ε-globin and ζ-globin). Interestingly, some of these genes downregulated by Myc encode transcription factors able to drive erythroid differentiation in cell culture, as well as in vivo models, such as GATA1, NFE2, STAT5A, STAT3, LMO2, LYL1, and JUNB. Moreover, Myc upregulated genes that block erythroid differentiation, such as the NOTCH ligand JAG2 and the transcription factor MA FK. Myc-mediated regulation of GATA1, NFE2, JUNB, and MA FK mRNA levels was confirmed by RT-qPCR (Fig. 6A). In contrast, Myc did not antagonize the p27-mediated downregulation of genes involved in DNA replication and mitosis, in agreement with the inability of Myc to
Myc inhibits p27-induced differentiation

Myc represses genes through the interaction with the zinc finger protein Miz1, forming a complex that binds to the region of the transcription initiation site (reviewed in reference 52). To test the possibility that Miz1 could be involved in the repression of erythroid genes and to confirm the effects of Myc on p27-induced differentiation, we transiently transfected K562 cells with expression vectors for p27, Myc, and MycV394D, a mutant unable to bind Miz1 (23). MycV394D was as efficient as wild-type Myc in antagonizing the upregulation of GATA1 and NFE2 induced by p27 (Fig. 6B). Consistently, we did not detect binding of Myc to the region of GATA1 gene that contains the transcription initiation site by chromatin immunoprecipitation (data not shown). This result suggests that Myc repress GATA1 and NFE2 through a Miz1-independent mechanism.

We also sought to determine whether the Myc effect is dependent on Myc box II. This region is required for transactivation, for transformation, and also for gene repression activities of Myc (reviewed in references 21 and 40). The results showed that MycD106-143 (carrying a deletion that encompasses Myc box II) was much less efficient at antagonizing p27-mediated upregulation of GATA1 and NFE2 (Fig. 6B) and other erythroid markers as ε-globin and EPOR (data not shown).

GATA1 antagonizes the effect of Myc as an inhibitor of erythroid differentiation. Of the genes regulated by Myc, we focused on GATA1 and NFE2, two transcription factors essential for erythroid lineage commitment (19). In erythroid differentiation, GATA1 nucleates the subsequent binding (2). Thus, we sought to determine whether GATA1 and NFE2 were partly responsible for the p27-induced differentiation by transiently transfecting Kp27MER cells. We first confirmed by immunoblot analysis that GATA1 transfection and overexpression (Fig. 6D) and benzidine staining (not shown). When both GATA1 and NFE2 were cotransfected, the effect was similar to that achieved by GATA1 alone (data not shown).

Myc antagonizes Mad1 upregulation mediated by p27. It has been shown that Mad1, which forms dimers with Max that repress transcription, antagonizes Myc functions. In addition,
FIG. 6. Erythroid genes downregulated by Myc. (A) Kp27MER and K562 cells were incubated for 12 h with ZnSO₄ and/or 4HT, and mRNA levels of the indicated erythroid transcription factor genes were determined by RT-qPCR. Values represent means ± the SEM of two independent experiments. (B) Expression of erythroid genes in K562 cells after transient transfection of vectors for p27, Myc, Myc- V394D (MycVD), and Myc D106-143 (MycD106). The plasmids were nucleofected, and the mRNA levels of GYP A, EPOR (24 h after transfection), or GATA1 and NFE2 (48 h after transfection) were determined by RT-qPCR. An expression vector for GFP was cotransfected in each case, and the data were normalized to the expression of GFP. Values are means ± the SEM from four independent experiments. (C) Enforced expression of GATA1 in Kp27MER. Cells were transfected by lipofection with GATA1 or empty vector. At 12 h after transfection, cells were treated with ZnSO₄ and/or 4HT. After 24 h of incubation, the expression of GATA1, p27, and Myc was analyzed by immunoblotting. The antibody against GATA1 used competes with the N-terminal domain of mouse protein. (D) GYP A mRNA levels assayed by RT-qPCR in Kp27MER cells transiently transfected with GATA1. At 12 h after transfection, the cells were treated for 24 h with ZnSO₄ and 4HT, and the mRNA level was determined by RT-qPCR. Values are means ± the SEM of two independent experiments. (E) ε-Globin mRNA levels of Kp27MER transfected with GATA1, assayed as in panel D. (F) Enforced expression of NFE2 in Kp27MER. Cells were transfected with an NFE2 expression vector or empty vector and analyzed by immunoblotting as in panel C. (G) ε-Globin mRNA levels of Kp27MER transfected with NFE2, assayed as in panel D. The data are means ± the SEM of two independent experiments.

DISCUSSION

One of the first biological activities described for Myc was differentiation inhibition, namely, the chemically induced erythroid differentiation of FMEL cells (8, 16, 44). This effect is consistent with the erythroleukemia induced in transgenic mice with enforced Myc expression in erythroid precursors (47). However, the mechanisms by which Myc blocks differentiation are poorly understood. Proliferation inhibition can certainly contribute to Myc-mediated inhibition of differentiation, but the hypothesis that Myc exerts proliferation-independent mechanisms to block differentiation has received little attention. Several studies have shown that Myc antagonizes the cell cycle arrest effect of p27, and the prevalent mechanism proposed is the sequestration of p27 in type D cyclin complexes (7, 43, 51). In contrast, there is little information on whether and how Myc impairs p27-induced differentiation. In this context, it has been shown that the monocytic differentiation induced by p27, and the prevalent mechanism is the sequestration of p27 in type D cyclins complexes. Furthermore, a transfection assay showed that Myc blocks the differentiation induced by p27 and inhibits the differentiation induced by Myc (15). Interestingly, transfection of p27 in K562 resulted in an increase in Myc mRNA (not shown). We next sought to determine whether Myc could contribute to p27-induced differentiation in this model. Cells were transiently transfected with expression vectors for Mad1 and Myc, and the erythroid differentiation was assayed by the benzidine test and the expression of ε-globin and EPOR. The results showed that Mad1 increased the expression of differentiation markers (Fig. 7A and B). We also transfected the MadMyc construct, which carries the transrepression domain of Mad1 linked to the HLH-LZ domain of Myc and acts as a strong repressor of Myc-responsive transcription (6). MadMyc was transfected into K562, and its expression demonstrated by immunoblotting. (Fig. 7A). We found that MadMyc efficiently induced differentiation as assessed by the upregulation of EPOR and ε-globin genes (Fig. 7E). Finally, coexpression of Myc blunted the differentiation induced by Mad1 and by MadMyc in transient-transfection experiments (Fig. 7C and D). We conclude that Mad1 upregulation is part of the differentiation response and that Myc also functions as a Mad1 antagonist in this system.

It has been reported that Mad1 induces erythroid differentiation in Friend murine erythroleukemia cells (10). Thus, we sought to determine whether the functional antagonism between Myc and Mad1 could also contribute to explain the Myc-mediated inhibition of differentiation in the K562 system. We assayed the expression of Mad proteins in the Kp27MER system and found that Mad1 protein (Fig. 7A) and mRNA (Fig. 7B) was upregulated upon p27-induced differentiation. Interestingly, Mad1 upregulation was reduced when Myc was activated by 4HT (Fig. 7A and B). In contrast, other members of the Mad family such as Mnt and Mxi1 were not upregulated by p27 (Fig. 7A). Moreover, transient transfection of p27 in K562 resulted in an increase in Myc mRNA (not shown). We next sought to determine whether Mad1 could contribute to p27-induced differentiation in this model. Cells were transiently transfected with expression vectors for Mad1 and Myc and the erythroid differentiation was assayed by benzidine test and the expression of ε-globin and EPOR. The results showed that Mad1 increased the expression of differentiation markers (Fig. 7A and B). We also transfected the MadMyc construct, which carries the transrepression domain of Mad1 linked to the HLH-LZ domain of Myc and acts as a strong repressor of Myc-responsive transcription (6). MadMyc was transfected into K562, and its expression demonstrated by immunoblotting. (Fig. 7C). We found that MadMyc efficiently induced differentiation as assessed by the upregulation of EPOR and ε-globin genes (Fig. 7E). Finally, coexpression of Myc blunted the differentiation induced by Mad1 and by MadMyc in transient-transfection experiments (Fig. 7C and D). We conclude that Mad1 upregulation is part of the differentiation response and that Myc also functions as a Mad1 antagonist in this system.

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FIG. 7. Mad1 induction by p27 and repression by Myc. (A) Immunoblot showing Mad1 expression in Kp27MER cells treated with 200 nM 4HT and the indicated concentrations of ZnSO4. The expression of p27, Mnt, and Mxi1 (members of the Mad family) was also determined. The levels of p21 and endogenous Myc are also shown to confirm the activation of Myc by 4HT. (B) Expression of Mad1 mRNA in Kp27MER and K562 cells. Cells were treated for 12 h with 4HT and ZnSO4, and mRNA expression was determined by RT-qPCR. The data are means ± the SEM of three experiments. (C) Mad1 induces the expression of erythroid markers in K562, and Myc impairs the Mad1-induced differentiation. K562 cells were nucleofected with expression vectors for Mad1, Myc, the MadMyc hybrid construct, and a mixture of Mad1+Myc and MadMyc+Myc vectors. At 24 h after transfection, the expression of the ectopic proteins was analyzed by immunoblotting. (D) K562 cells were transfected as in panel C, and the benzidine-positive cells were scored 72 h after transfection to assess erythroid differentiation. The data are means ± the SEM of two independent experiments. (E) EPOR and ε-globin mRNA levels were assayed by RT-qPCR in K562 cells transiently transfected as described in panel C. The data are means ± the SEM of two independent experiments.

not entirely depend on cell cycle arrest since p21 also arrests proliferation of K562 while inducing a different type of differentiation (34). In the present study we show that Myc impairs the erythroid differentiation through a mechanism that is independent of the proliferation arrest and Cdk inhibition imposed by p27. Thus, the Myc effect on differentiation inhibition is uncoupled from its effects on proliferation. Microarray analysis showed that Myc antagonizes the p27-mediated upregulation of genes of transcription factors that direct erythroid differentiation (GATA1, NFE2, JUNB, LMO2, and STAT5A). Myc also antagonizes the p27-mediated downregulation of genes that oppose erythroid differentiation (MAFK and JAG2). Moreover, enforced expression of GATA1, a “master gene” of erythropoiesis (19), partially rescues the differentiation inhibition induced by Myc, indicating that Myc effect is mediated, in part, by antagonizing GATA1 induction. However, GATA1 is only partly rescuing the anti-differentiation effect of Myc, since Myc is likely impairing differentiation by repressing several erythroid-determining genes.

The molecular mechanism by which Myc represses GATA1 is unclear. Actually, about half of Myc target genes are repressed, but the mechanism of repression is unknown for the vast majority of the genes. In a small subset of genes, Myc-mediated repression depends on the interaction with Miz1 (52). In the case of GATA1 in the K562 model, this effect seems to be Miz1 independent, since a Myc mutant unable to bind Miz1 antagonized the upregulation of GATA1 and erythroid differentiation as efficiently as did wild-type Myc. In contrast, a Myc mutant lacking Myc box II (a conserved region required for transformation, transactivation, and repression activities of Myc) cannot efficiently antagonize p27-induced differentiation in our model. This result has also been observed for other differentiation models (4, 20) and suggests that Myc is using a common molecular mechanism to transform and to abrogate differentiation. On the other hand, it has been reported that GATA1 represses Myc and induces p27 in murine embryonic cells (45), suggesting the possibility of a regulatory p27-GATA1 loop. However, GATA1 does not repress Myc in our K562 model (data not shown).

Myc and Mad1 have antagonist activities in transcription upon binding to the same E-boxes and in cell proliferation (5, 21). We found that p27-induced differentiation of K562 is accompanied by Mad1 upregulation and that Mad1 is able to promote K562 erythroid differentiation, although less efficiently than p27. Moreover, MadMyc, a chimeric protein that represses gene expression upon binding to Myc-binding sites (6), is a potent differentiation inducer. Thus, Myc would antagonize this Mad1 effect at the transcriptional level. Furthermore, Myc activation in our model provokes Mad1 downregulation in K562 cells and Myc coexpression impairs Mad1 and MadMyc-induced differentiation. This suggests that the impairment of Mad1 function by Myc is contributing to differentiation inhibition in the K562p27MER model. These results are consistent with Mad1 inducing erythroid differentiation of murine erythroleukemia cells (10). Moreover, it has been shown that Mad1 and p27 cooperate for a correct development of the myeloid compartment in vivo (33). Similar scenery seems to operate in K562 differentiation, and our data suggest that Myc might impair the Mad1-p27 functional interaction.

In summary, the results in the K562 model demonstrate that the differentiation-inhibitory effect of Myc depends on its gene regulatory activity and is uncoupled from its effect as proliferation stimulator. Importantly, tumor cell redifferentiation is the mechanism for the tumor regression after Myc deactivation in some transgenic mice models (reviewed in reference 3).
Moreover, Myc is one of the four transcription factor set capable of conferring pluripotent stem cell properties to differentiated adult cells, and Myc is reactivated in the tumors arising in the chimeric mice derived from such cells (38). Thus, it is conceivable that Myc displays common pathways as a “stem cell-ness” keeper and as an oncogene, preventing differentiation in both cases. Our results in the K562 model support the hypothesis that inhibition of cell differentiation is a Myc tumorigenic mechanism independent of Myc effects in cell proliferation.

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