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c-Myc depletion inhibits proliferation of human tumor cells at various stages of the cell cycle

H Wang[1], **S Mannava**[2], **V Grachtchouk**[2], **D Zhuang**[2], **MS Soengas**[2], **AV Gudkov**[3], **EV Prochownik**[1,4], and **MA Nikiforov**[2,4]

[1]Section of Hematology/Oncology, Children's Hospital of Pittsburgh, Rangos Research Center, Pittsburgh, PA, USA

[2]Department of Dermatology, Comprehensive Cancer Center, University of Michigan, Ann Arbor, MI, USA

[3]Department of Molecular Genetics, Cleveland Clinic, Lerner Research Institute, Cleveland, OH, USA

Abstract

A major role for c-Myc in the proliferation of normal cells is attributed to its ability to promote progression through G_1 and into S phase of the cell cycle. The absolute requirement of c-Myc for cell cycle progression in human tumor cells has not been comprehensively addressed. In the present work, we used a lentiviral-based short hairpin RNA (shRNA) expression vector to stably reduce c-Myc expression in a large number of human tumor cell lines and in three different types of normal human cells. In all cases, cell proliferation was severely inhibited, with normal cells ultimately undergoing G_0/G_1 growth arrest. In contrast, tumor cells demonstrated a much more variable cell cycle response with cells from several lines accumulating in S or G_2/M phases. Moreover, in some tumor lines, the phase of cell cycle arrest caused by inhibition of c-Myc could be altered by depleting tumor suppressor protein p53 or its transcriptional target $p21^{CIP/WAF}$. Our data suggest that, as in the case of normal cells, c-Myc is essential for sustaining proliferation of human tumor cells. However its rate-limiting role in cell cycle control is variable and is reliant upon the status of other cell cycle regulators.

Keywords

Myc; cell cycle; shRNA

Introduction

By virtue of its central role as a general transcription factor, the c-Myc oncoprotein regulates a number of key normal cellular processes such as growth, proliferation, cell cycle progression, apoptosis and differentiation (Grandori *et al.*, 2000; Adhikary and Eilers, 2005; Meyer *et al.*, 2006). The importance of c-Myc is underscored by the fact that its inhibition leads to cell cycle arrest *in vitro* and early embryonic lethality *in vivo* (Prochownik *et al.*, 1988; Trumpp *et al.*, 2001; Zhou and Hurlin, 2001; Lutz *et al.*, 2002). Furthermore,

Correspondence: Dr MA Nikiforov, Department of Dermatology, University of Michigan, 1150 W Medical Center Drive, MS1 Room. 6427, Ann Arbor, MI 48109, USA. mikhailn@umich.edu.

[4]These authors contributed equally to this work.

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deregulated overexpression of c-Myc, either *in vitro* or *in vivo*, leads to malignant transformation (Grandori *et al.*, 2000; Lutz *et al.*, 2002). Taken together, these data suggest that the accumulation of c-Myc mRNA and protein in numerous and diverse human tumors plays a role in the pathogenesis of these neoplasms (Nesbit *et al.*, 1999).

The critical importance of c-Myc as a transforming oncogene has led to a number of strategies designed to inhibit its expression or function (Prochownik, 2004; Ponzielli *et al.*, 2005; Vita and Henriksson, 2006). These efforts have been fueled, in part, by experimental systems showing that tumor cell proliferation and/or survival rely upon sustained expression of c-Myc (Adhikary and Eilers, 2005). Despite these findings, the idea that all cells have an obligate requirement for c-Myc has recently been challenged. For example, somatic knockout of both alleles of *c-myc* in spontaneously immortalized rat fibroblasts resulted in a several-fold reduction of proliferation but not in its complete inhibition (Mateyak *et al.*, 1997). In liver, the conditional inactivation of *c-myc* impairs hepatocyte proliferation in neonatal but not adult mice (Baena *et al.*, 2005), although another report suggested that proliferation of adult hepatocytes is also *c-myc*-independent (Li *et al.*, 2006). Accordingly, conditional inactivation *c-myc* did not affect self-renewal of intestinal mucosa in juvenile and adult mice (Bettess *et al.*, 2005). Additionally, *c-myc* was found to be dispensable for proliferation of mouse skin keratinocytes *in vivo*, but not for their transformation by activated H-Ras (Oskarsson *et al.*, 2006). Based on these results, it is conceivable that the proliferation of certain types of cells occurs via c-Myc-independent pathways. In accord with this suggestion, we and others have demonstrated that resumption of normal proliferation as well as other aspects of c-Myc phenotype can be imparted to *c-myc* knockout fibroblasts by overexpression of individual downstream c-Myc target genes (Hermeking *et al.*, 2000; Nikiforov *et al.*, 2002; Rothermund *et al.*, 2005). Together, these results raise the formal possibility that, due to their genetic heterogeneity, some tumor cells may overcome their dependency on c-Myc by expressing the proper combination of surrogate genes. Clearly, determining the extent to which this is true is critical before serious consideration can be given to the use of c-Myc inhibitors in the clinical setting.

In normal cells, inhibition of c-Myc invariably results in a G_0/G_1 cell cycle arrest (Prochownik *et al.*, 1988; Mateyak *et al.*, 1997; de Alboran *et al.*, 2001; Prathapam *et al.*, 2006). The type of cell cycle arrest caused by c-Myc depletion in tumor cells has not been adequately studied. Data available to date on this subject were collected on small number of lines (Skorski *et al.*, 1995; McGuffie *et al.*, 2000; Chen *et al.*, 2001; Hermeking, 2003) and the methods utilized for inhibiting c-Myc were not consistent (D'Agnano *et al.*, 2001; Citro, 1998). Therefore, the extent to which tumor cells resemble normal cells with regard to the type of cell cycle arrest, if any, caused by c-Myc depletion remains unclear.

In the current report, we have for the first time undertaken a comprehensive study of the phenotypes caused by c-Myc depletion in a large number of human tumor cell lines (22) and in three reference normal human cell types (fibroblasts, keratinocytes and melanocytes). For this purpose, we utilized a standardized and specific method of reducing endogenous c-Myc levels, for example, lentivirus-mediated delivery of short hairpin c-Myc RNAs (Myc-shRNAs). This approach was able to provide stable inhibition of c-Myc in nearly 100% of cells. As a result of these studies, we have demonstrated that the requirement for c-Myc to maintain proliferation is absolute in all examined cells, suggesting that this could be generally true. However, unlike normal cells, which invariably arrest in G_0/G_1 phase of the cell cycle in response to c-Myc depletion, tumor cells exhibit significant heterogeneity with regard to the positioning of cell cycle arrest. As demonstrated here, this heterogeneity could be dependent upon the intactness and relative importance of other cell cycle control pathways.

Results

Selection of human c-Myc-specific shRNA

To determine whether tumor cell proliferation depends on c-Myc, we depleted its endogenous levels using lentivirus-delivered shRNAs. As a prelude, several candidate Myc-shRNAs were cloned into lentiviral vector H1, which also encoded enhanced green fluorescent protein (eGFP) (Ivanova *et al.*, 2006). In addition, a set of commercially available human c-Myc-specific shRNAs in the pLKO1 vector was also tested. The specificity and effectiveness of these shRNAs were first tested in immortalized *c-myc*-null rat fibroblasts (HO15.19) (Mateyak *et al.*, 1997) reconstituted with human c-Myc cDNA (HO15.19-h-c-Myc). The 'myc-null' phenotype has been extensively characterized in HO15.19 cells, and therefore these cells serve as an excellent reference line in which to study shRNA-mediated inhibition of c-Myc expression. Supplementary Figure S1 summarizes features of HO15.19-h-c-Myc cells expressing the most effective Myc-shRNAs (M1, M2, M3). In each case we observed efficient inhibition of c-Myc as evidenced by western blotting, characteristic changes in cell morphology of Myc-depleted HO15.19-h-c-Myc cells and an increase in the fraction of cells in G_0/G_1 phase of the cell cycle. We therefore used these validated shRNAs for inhibition of c-Myc in human cells.

Inhibition of proliferation of normal and tumor cells by Myc-shRNAs

We studied 22 human tumor cell lines originating from a wide variety of adult and pediatric cancers (Table 1). Normal control cells consisted of primary human fore-skin keratinocytes, melanocytes and fibroblasts. All cells were infected with control shRNA or Myc-shRNAs as described in Materials and methods. The efficiency of infection was quantified by counting GFP-positive cells (which was always ≥95%, not shown). When possible, the degree of c-Myc inhibition was monitored within 48–96 h after infection by western blotting (Figure 1a, Supplementary Figure S2 and data not shown). Our analysis revealed that Myc-suppressing activities of M1, M2 and M3 varied significantly among different lines (data not shown). Therefore, based on preliminary experiments, we utilized Myc-shRNAs, which provided the most efficient inhibition of c-Myc in a given cell line.

In order to evaluate the impact of Myc-shRNAs expression on cell proliferation, cells were plated at low density 2 days after infection and were counted and/or photographed during the ensuing 6 days. As seen in Figure 1b and Supplementary Figure S3, all cells infected with control lentiviral continued to proliferate logarithmically. In contrast, cells infected with the Myc-shRNAs showed an abrupt cessation of proliferation starting at day 3 or 4 post infection. In some cases, cells underwent apoptotic death starting as early as day 4 post infection (Supplementary Figure S4).

In several cases, we observed the eventual emergence of proliferating cells from Myc-shRNA-infected cultures. Western blot analysis revealed that c-Myc expression, while initially low, had returned to the pre-infection levels of control cells (not shown). This suggested that these cells had somehow escaped Myc-shRNAs action or that the efficiency of infection of these cells was suboptimal, thus allowing for the outgrowth of cells with a proliferative advantage. We therefore conclude that the continuous suppression of c-Myc is mandatory for the proliferative arrest of all cell lines examined.

Cell line-specific effects of c-Myc depletion

A large body of literature suggests that inhibition of c-Myc in primary cells or non-transformed cell lines generally leads to a strong proliferative arrest in the G_0/G_1 or G_1/S phases of the cell cycle (Prochownik *et al.*, 1988; Mateyak *et al.*, 1997; de Alboran *et al.*, 2001; Prathapam *et al.*, 2006). In order to determine whether this is also the case for tumor

cells, we used propidium iodide (PI) staining and flow cytometry to evaluate the cell cycle parameters of the above-described cells 4–5 days after infection (Figure 2, Supplementary Figure S5). In parallel, control and two Myc-shRNAs were introduced separately into populations of normal skin melanocytes, fibroblasts and keratinocytes. Overall, the cell cycle profiles of cells infected with control lentiviral vector did not differ significantly from those of their uninfected counterparts (not shown). In normal cells, depletion of c-Myc resulted in G_0/G_1 growth arrest, as previously reported (Prochownik *et al.*, 1988; Mateyak *et al.*, 1997; de Alboran *et al.*, 2001; Prathapam *et al.*, 2006). In contrast, tumor cells expressing Myc-shRNAs displayed several distinct types of cell cycle arrest as depicted in Figure 2 and Supplementary Figure S5. For example, similar to normal melanocytes, melanoma lines SK-Mel-19 and SK-Mel-29 arrested in G_0/G_1 in response to c-Myc depletion. In contrast, c-Myc depletion in melanoma cells UACC62 and UACC257 resulted in S-phase arrest accompanied by apoptosis at days 4–5 (Supplementary Figure S4), whereas SK-Mel-28 melanoma cells and HT1080 osteosarcoma cells arrested in G_2/M without any discernible apoptosis. In further contrast, c-Myc inhibition in SK-Mel-94and Malme 3M melanoma cells resulted in cell cycle arrest concurrently in G_0/G_1 and G_2/M phases. In colon cancer line HCT-116, cell cycle profiles of Myc-depleted cells were nearly identical to those of cells infected with the control vector. Since these cells demonstrated dramatically reduced proliferation rates (Supplementary Figure S3B), we conclude that c-Myc depletion blocks cell cycle progression in these cells equally in all phases. The cell cycle profiles of all other cancer lines were basically similar to one of those described above (Table 1, Supplementary Figure S5). Independent infection of a number of these lines with another c-Myc-shRNA lentivirus produced similar results thus indicating that the cell cycle arrest was truly a specific consequence of c-Myc depletion (not shown).

Our findings indicate that the cell cycle response to c-Myc depletion in normal cells is invariably associated with G_0/G_1 arrest. However, among tumor cell lines, considerable cell cycle heterogeneity is observed, even among lines derived from identical tumor types. These observations suggest that other factors, whose expression levels or mutational status differ between normal and tumor cells, determine the type of cell cycle arrest in c-Myc-depleted tumor cells.

Inhibition of p53 or p21 alters the cell cycle profile of c-Myc-depleted cells

We were interested in understanding the molecular mechanisms that allow cells from several tumor lines to escape the G_0/G_1 growth arrest that characterizes c-Myc-depleted normal cells. It is well accepted that the expression of factors promoting G_0/G_1 arrest in response to different forms of stress can be altered in tumor cells (Hahn and Weinberg, 2002). Therefore, we hypothesized that qualitative or quantitative differences in such factors might underlie the different cell cycle profiles of c-Myc-depleted normal and tumor cells. To test this hypothesis, we first investigated the impact of inhibiting the p53 tumor suppressor protein on the cell cycle parameters of c-Myc-depleted cells undergoing G_0/G_1 arrest. We focused on p53, since it is involved in the G_1/S checkpoint and because its inactivation or loss occurs frequently in tumor cells (Hanahan and Weinberg, 2000). To this end, we chose melanoma lines SK-Mel-19, -29, -103 and -147, which express wild-type p53 (Soengas *et al.*, 2001), and which arrest in G_0/G_1 in response to c-Myc depletion (Figure 2). Primary melanocytes were also included in the experiment to compare cell cycle responses between normal and tumor cells of similar origin. As shown in Supplementary Figure S6, depletion of c-Myc resulted in slight increase in p53 levels in SK-Mel-19 and SK-Mel-29 cells and in moderate increase in p53 levels in SK-Mel-103 and SK-Mel-147 cells: 1.5–2 fold and 2.4–3.3 fold, respectively. Simultaneous suppression of p53 and c-Myc (by M1 shRNA) (Figure 3a) led to differences in the cell cycle arrest profiles among the studied cells (Figure 4). In normal melanocytes and cells from lines SK-Mel-19 and SK-Mel-29, G_0/G_1 phase was

increased by 17, 30 and 32%, respectively, in response to c-Myc depletion only and by 25, 49 and 50%, respectively in cells depleted of both c-Myc and p53 (Figure 4). Depletion of c-Myc alone in melanoma cells SK-Mel-103 and SK-Mel-147 also resulted in corresponding increases of G_0/G_1 phase by 17 and 21%. In contrast, concurrent c-Myc and p53 depletion in these cells, rather than increasing the G_0/G_1 population actually caused modest declines of 17 and 11%, respectively and concomitant increases in the G_2/M populations by 85 and 92%, respectively (Figures 3a and 4). Similar results were obtained with M2 shRNA (shown for SK-Mel-103 cells in Supplementary Figure S7). These data suggest that p53 levels could indeed influence the cell cycle consequences of c-Myc depletion in at least some tumor cell lines. Of note was that all c-Myc-depleted cells, irrespective of their p53 levels and wherein the cell cycle arrest occurred, showed total inhibition of proliferation (Figure 3b). This suggested that suppression of p53 is not sufficient to rescue the proliferative deficiencies caused by c-Myc depletion.

One of the major mediators of p53-directed cell cycle arrest is the cyclin-dependent kinase inhibitor (CDKI) $p21^{CIP/WAF}$ (El-Deiry *et al.*, 1993). We therefore assessed its role in mediating G_0/G_1 growth arrest induced by c-Myc depletion. As shown in Figures 3 and 4, inhibition of $p21^{CIP/WAF}$ in all cell lines resulted in patterns of cell cycle arrest similar to, although less pronounced than those observed in p53-depleted cells.

Taken together, our data suggest that c-Myc is essential for proliferation of all tested normal and tumor cells. However, the rate-limiting function of c-Myc in controlling cell cycle progression is cell line-dependent and is variably reliant upon the status of other cell cycle checkpoint regulators, even within tumors originating from the same cell type.

Discussion

The widespread deregulation of c-Myc in many tumor types together with its role in maintaining the transformed state in certain experimental settings (Arvanitis and Felsher, 2006) has provided the rationale for its consideration as a prime therapeutic target. Indeed, a number of novel strategies have already been employed to inhibit c-Myc expression or function in tumor cells (Hermeking, 2003; Prochownik, 2004; Ponzielli *et al.*, 2005). In several transgenic mouse models of c-Myc-mediated tumorigenesis, abolishing c-Myc overexpression has been shown to lead to complete tumor regression (Felsher and Bishop, 1999; Pelengaris *et al.*, 1999, 2002; Jain *et al.*, 2002). In contrast to these reports, c-Myc appears to be dispensable for the maintenance of mouse mammary gland tumors (Boxer *et al.*, 2004). It is also noteworthy that in all the above mouse models, *c-myc* was an initiating oncogene leading to transformation, whereas this is unlikely to be the case with the majority of spontaneously arising human cancers. Therefore, the absolute requirement of c-Myc for proliferation of tumor cells in general and human tumor cells in particular still remains an open question.

The studies reported here, performed with a variety of human tumor cell lines, indicate that they are all highly susceptible to Myc-shRNA-mediated growth inhibition. Indeed, some cell lines demonstrated marked growth arrest even when c-Myc ablation was less than complete (70–80% inhibition), thereby suggesting that human cancers could be sensitive to anti-c-Myc therapies that only partially reduce c-Myc levels or function. On the other hand, it was reported that loss of c-Myc did not inhibit proliferation of normal mouse hepatocytes (Baena *et al.*, 2005; Li *et al.*, 2006), keratinocytes (Oskarsson *et al.*, 2006) and intestinal epithelium (Bettess *et al.*, 2005) *in vivo*. There are several possible explanations for the difference between our data and those of Baena *et al.* (2005); Oskarsson *et al.* (2006) and Li *et al.* (2006). The first one is that in some cases proliferation of c-Myc-depleted cells could continue to depend on the expression of other Myc family members, including N-Myc,

which has been shown to functionally compliment c-Myc in *in vivo* settings (Malynn *et al.*, 2000). The second explanation is that expression of c-Myc downstream targets that are critical for the proliferation of the above cells in mouse could depend on the presence of cytokines or hormones (such as, for example, hedgehog protein for skin development (Athar *et al.*, 2006) that are absent in the media routinely used in tissue culture. Finally, it is possible that proliferation of human tumor cells is more dependent on c-Myc than that of normal mouse cells, a phenomenon known as oncogene 'addiction' (Sharma *et al.*, 2006).

The requirement of c-Myc for normal cell cycle progression has been extensively studied in primary or immortalized rodent cells where c-Myc inactivation results in severe or complete inhibition of proliferation and growth arrest predominantly in G_0/G_1 (Prochownik *et al.*, 1988; Mateyak *et al.*, 1997; de Alboran *et al.*, 2001; Prathapam *et al.*, 2006). Our data obtained in three different types of primary human cells are in good agreement with the majority of the above reports. On the other hand, while also achieving complete proliferative arrest in tumor cells following c-Myc depletion, we observed considerable variability in cell cycle consequences.

Tumor progression is often accompanied by mutations affecting genes whose products control G_1/S transition (Hanahan and Weinberg, 2000). Therefore, the mechanisms involved in implementing G_0/G_1 growth arrest in response to c-Myc depletion present in normal cells could be impaired in tumor cells. Depending on the nature of these defects, c-Myc depletion would affect G_1/S transition differently, even in tumors of the same type as shown in the current work. Consequently, due to the involvement of c-Myc in multiple cellular processes (Eisenman, 2001; Patel *et al.*, 2004), its inhibition may result in activation of other cell cycle checkpoints downstream to G_1/S, including those involving S (Gottifredi and Prives (2005) or G_2/M (Stark and Taylor, 2006).

We demonstrated that interference with levels of p53 and its downstream CDKI target $p21^{WAF/CIP}$ in two melanoma lines (SK-Mel-103 and SK-Mel-147) permits them to transit G_1/S and arrest instead in G_2/M, without any apparent reversal of their proliferative defect. In contrast, suppression of p53 or $p21^{CIP/WAF}$ in normal melanocytes or in melanoma cells SK-Mel-19 and SK-Mel-29 resulted only in a more marked G_0/G_1 growth arrest. Accordingly, induction of p53 by c-Myc-depletion was more pronounced in SK-Mel-103 and SK-Mel-147 compared to SK-Mel-19 and SK-Mel-29 cells (Supplementary Figure S6). Interestingly, an observed increase in p53 levels (at least in SK-Mel-103 and SK-Mel-147 cells) is unlikely to be dependent on $p14^{ARF}$ since this gene is deleted in both of these cell lines (Paz *et al.*, 2003). The simplest explanation for the difference in p53 or $p21^{CIP/WAF}$ dependency of G_0/G_1-growth arrest induced by c-Myc-depletion in studied cells is that, in addition to p53 and $p21^{CIP/WAF}$, multiple proteins are involved in the implementation of G_0/G_1 arrest caused by c-Myc inhibition. According to our model, their mutational status and/or expression levels are not altered in normal melanocytes and in melanoma cells SK-Mel-19 and SKMel29 but are altered in SK-Mel-103 and SK-Mel-147 cells.

It is conceivable that G_0/G_1 arrest caused by c-Myc inhibition could occur *via* at least two non-mutually exclusive mechanisms. The first could involve deregulated expression of c-Myc-dependent checkpoint genes (CDKs and CDKIs, such as cdk4 and $p27^{KIP1}$; Bernard and Eilers, 2006; Dang *et al.*, 2006). Alternatively, c-Myc inactivation could lead to changes in the pools of specific cellular metabolites, since c-Myc directly regulates genes whose products play key roles in several metabolic pathways (Dang, 1999). The consequences of these alterations could be growth arrest at different stages of the cell cycle depending on the status of other key cell cycle regulators. In accord with the latter suggestion are the findings that depletion of the pyrimidine nucleotide pool in normal fibroblasts, achieved by chemical inhibition of the direct c-Myc target CAD (Carbamoyl-phosphate synthetase 2, Aspartate

transcarbamylase, Dihydroorotase), results in G_0/G_1 growth arrest. Interestingly, similar inhibition of CAD in p53-null fibroblasts also results in growth arrest, although in S-phase rather than G_0/G_1 (Agarwal *et al.*, 1998).

In conclusion, our findings regarding the absolute reliance of tumor cells on endogenous levels of c-Myc could be important when considering therapeutic approaches that aim to target this oncoprotein. The considerable differences in the cell cycle responses to c-Myc depletion noted here also point to yet additional heterogeneity at the level of other cell cycle regulators, even within tumors arising from the same tissues.

Materials and methods

Tumor cell lines

A list of the tumor cell lines used is provided in Table 1. Cell lines were originally obtained from the ATCC or from Memorial Sloan Kettering Cancer Center. Cells were cultured in either RPMI-1640 or Dulbecco's modified Eagle's essential minimal medium as recommended by the supplier. Supplements included fetal calf serum (10–20%), 2 m_M glutamine and 100 U ml^{-1} penicillin G + 100 μg ml^{-1} streptomycin. All cell culture agents were purchased from Invitrogen Inc. (Carlsbad, CA, USA). Primary fibroblasts, melanocytes and keratinocytes were obtained from neonatal foreskin and maintained as described before (Fernandez *et al.*, 2005; Denoyelle *et al.*, 2006).

Plasmids and transfections

The lentiviral vector containing shRNA specific for p53 was described before (Boiko *et al.*, 2006). The lentiviral vectors containing an shRNA specific for human $p21^{CIP/WAF}$ and for human c-Myc were purchased from Sigma. The lentiviral construct H1 (Ivanova *et al.*, 2006) was used to express shRNAs directed against human c-Myc. The c-Myc targeting sequences consisted of nt. 1567–1585 (M1), 1341–1359 (M2) and 1919–1937 (M3) of the previously published sequence (GenBank Accession no. NM_002467.3). A control lentiviral vector contained an irrelevant sequence. Lentiviral packaging reactions were performed in the 293-FT cell line in the presence of packaging plasmids pVSV-G and pΔDR (Ivanova *et al.*, 2006) using Superfect Transfection Reagent (Qiagen Inc., Chatsworth, CA). Viral supernatants were collected 48 h after transfection, filtered through disposable 0.45 μ_M cellulose acetate filters (VWR Scientific Inc. West Chester, PA, USA) and frozen in individual aliquots at −80 °C. For infection cells were plated in 60 or 100 mm tissue culture dishes (VWR) and allowed to achieve 40–50% confluence before adding viral supernatant in the presence of 8 μg ml^{-1} polybrene for 24h (Sigma, St Louis, MO, USA). Cells infected with H1 lentiviral vector expressing different shRNAs were examined by fluorescence microscopy for the expression of EGFP in 48 h. Cells were then split and cultured for an additional 2–4 days after infection before being utilized for further studies.

Immunoblotting, immunohistochemistry and flow cytometry

Total cell lysates were prepared and immunoblotting was performed as previously described using 50–80 μg of total protein (48). The following antibodies were used: 9E10 and C33 monoclonal antibody (mAb) for human c-Myc (SC-40, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), p53 (VP-P955, Vector Laboratories Ltd, Peterborough, UK) and $p21^{CIP/WAF}$ (SC-397), H2AX-Ser139-phospho-specific antibodies (Millipore Corporation, Billerica, MA, USA), D-10 mAb against tubulin (Santa Cruz Biotechnology). Membranes were developed using enhanced chemiluminescence kit (Super-Signal West Femto Maximum Sensitivity Substrate, Pierce, Rockland, IL, USA) or using alkaline phosphatase secondary antibodies and detection/quantification of the signal was performed using ImageJ 1.38 software or STORM Phosphor-Imager and ImageQuant 2.0 Program as a part of

ImagePro Analysis Software package. Background was calculated from an equivalent area in each lane and subtracted from the value for c-Myc in that lane. Propidium iodide staining, flow cytometry and statistical evaluation of cell cycle data were performed as previously described (Nikiforov *et al.*, 2002; Yin *et al.*, 2002).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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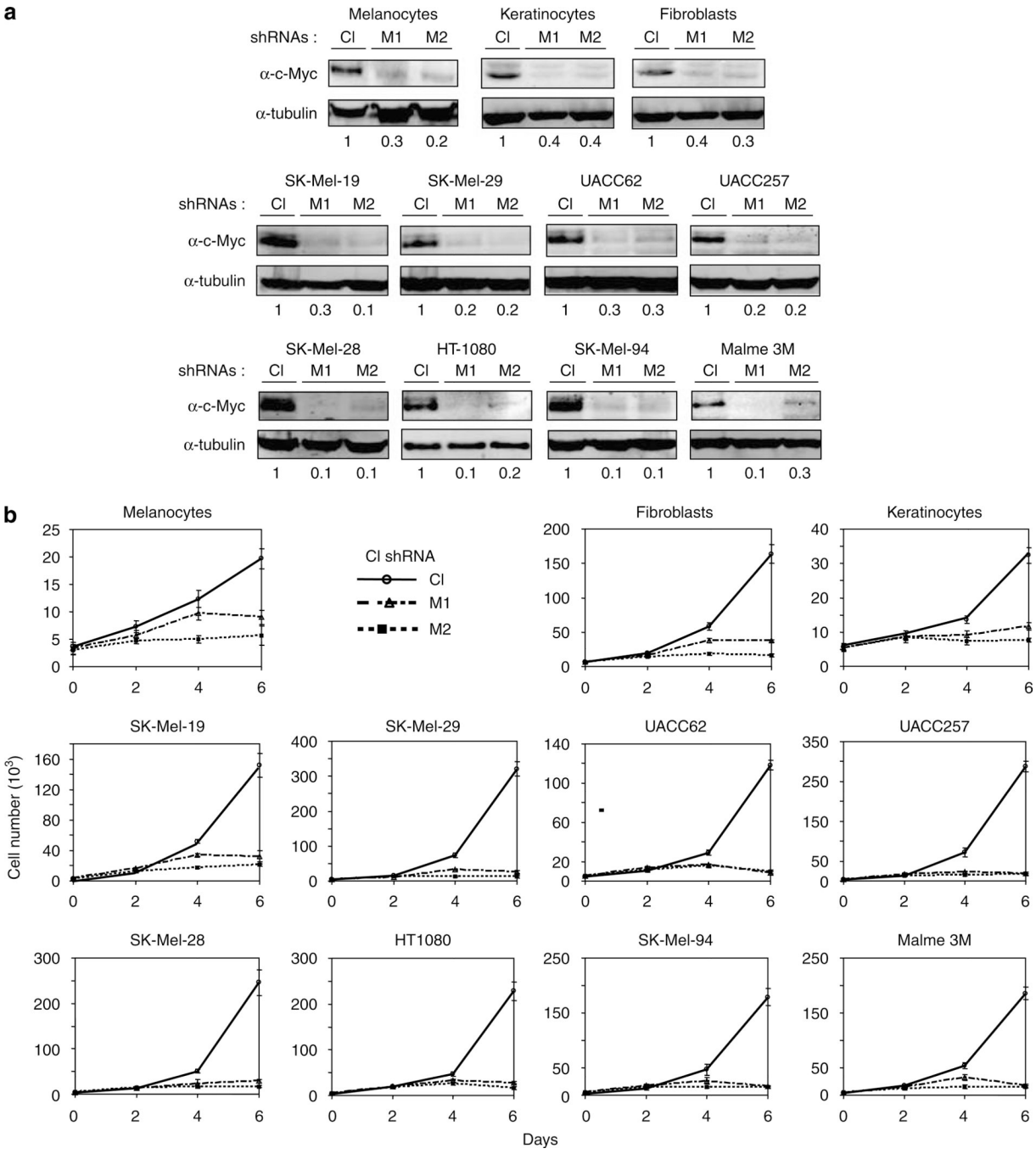

Figure 1.

Inhibition of c-Myc expression in normal and tumor human cells leads to arrest of proliferation. Each of the indicated tumor cell lines or populations was infected during log-phase growth with lentiviral vectors encoding a control shRNA (Cl) or two different c-Myc-shRNAs M1 or M2. (**a**) Cells were harvested at 4days post infection in the SDS-containing buffer, resolved on 8% polyacrylamide gel, transferred to PVDF membranes and probed in western blotting with the antibodies designated on the right. Numbers below the panels represent c-Myc amounts normalized by the amounts of tubulin and amounts of c-Myc in control samples. (**b**) Tumor cells were plated in triplicates in 24-well plates (2×10^3 cells per well), normal cells were plated in triplicates in 24-well plates ($0.5–2 \times 10^4$ cells per

well). Cells were counted every other day for 6 days. Numbers below the graph indicate days post infection.

Figure 2.

c-Myc depletion causes different types of cell cycle arrest in normal and tumor cells. The indicated cell lines were infected during log phase growth with lentiviral vectors expressing either control (Cl) or c-Myc M1 or M2 shRNAs. Four days post infection the subconfluent cultures were harvested, stained with propidium iodide and analysed by flow cytometry. See Supplementary Figure S5 for the cell cycle profiles of other cell lines.

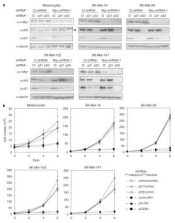

Figure 3.

Inhibition of p53 or $p21^{CIP/WAF}$ does not compensate for the depletion of c-Myc. Cells from the indicated lines were infected with lentiviral vectors expressing control − p53 −or $p21^{CIP/WAF}$-shRNAs as described in Materials and methods. Two days later, cells were superinfected with control or c-Myc shRNA (M1). (**a**) Cells were harvested 4days after the second infection and examined by western blotting with the antibodies designated on the right. ShRNA′ and shRNA″ designate shRNAs used for primary and secondary infections, respectively. Note a background band in melanocyte cell extracts probed with p53 antibodies closely migrating with the correct band, designated with the arrow. (**b**) Cells were plated into 12-well plates and counted daily in triplicates in the presence of trypan blue.

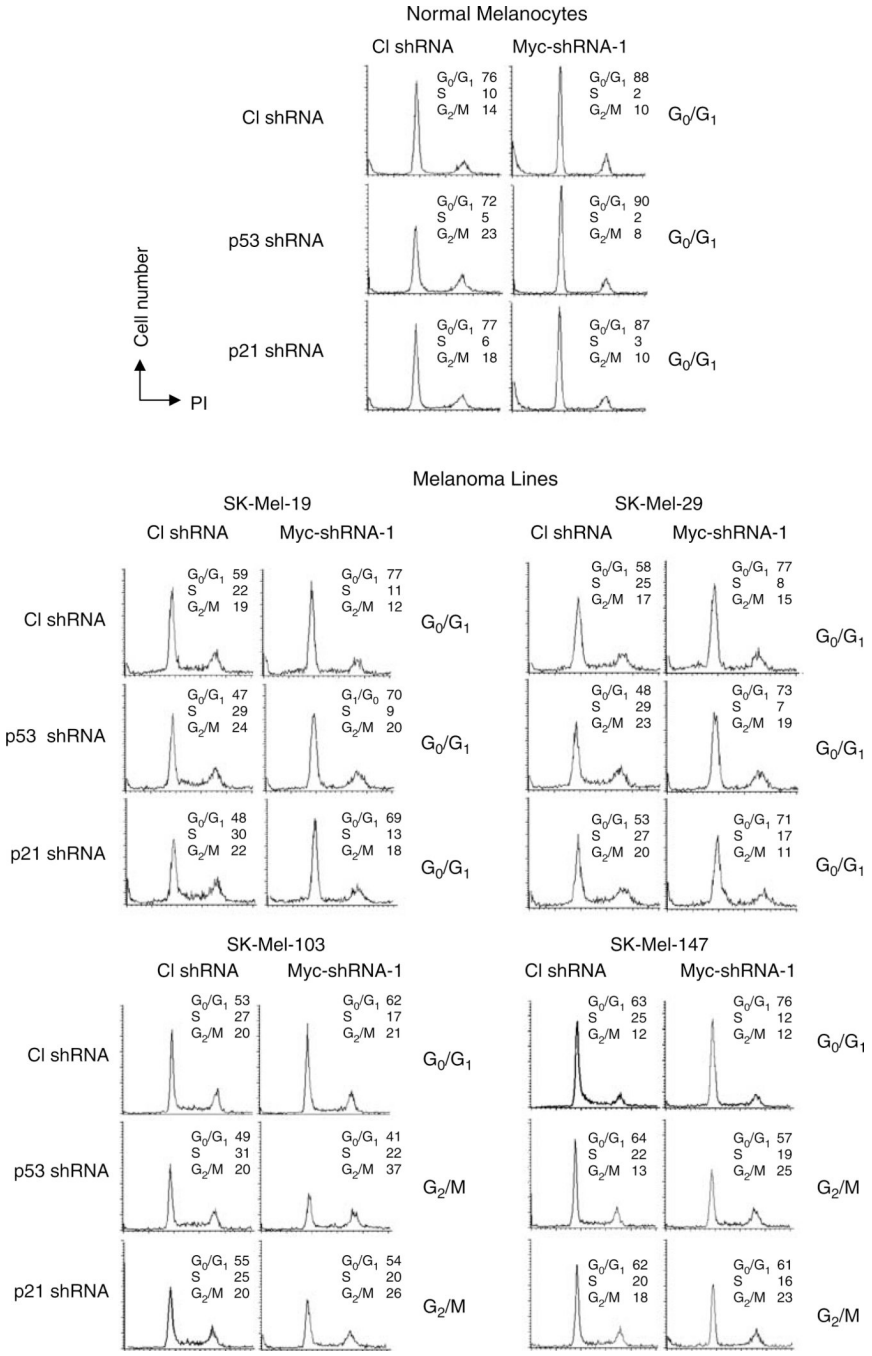

Figure 4.

Cell cycle parameters of c-Myc-depleted cells are altered by inhibition of p53 or $p21^{CIP/WAF}$. Cells from the indicated lines were infected with lentiviral vectors expressing control − p53 − or $p21^{CIP/WAF}$-shRNAs as described in Materials and methods. Two days later, cells were superinfected with control or Myc shRNA (M1) lentiviral vectors. Cells were collected for cell cycle analysis 4days after the second infection.

Table 1

Normal and tumor cells utilized in the study

#	Cell line	Type of tissue/tumor	Cell cycle arrest[a]
1	Melanocytes	Skin	G_0/G_1
2	Keratinocytes	Skin	G_0/G_1
3	Fibroblasts	Skin	G_0/G_1
4	Mel-1 9	Metastatic melanoma	G_0/G_1
5	SK-Mel-28	Metastatic melanoma	G_2/M
6	SK-Mel-29	Metastatic melanoma	G_0/G_1
7	SK-Mel-94Metasta	Metasta tic melanoma	$G_0/G_1+G_2/M$
8	SK-Mel-103	Metastatic melanoma	G_0/G_1
9	SK-Mel-147	Metastatic melanoma	G_0/G_1
10[b]	UACC62	Metastatic melanoma	S
11[b]	SK-Mel-173	Metastatic melanoma	G_0/G_1
12	Malme 3M	Metastatic melanoma	$G_0/G_1+G_2/M$
13[b]	UACC257	Metastatic melanoma	S, G_2/M
14	SAOS-2	Osteosarcoma	G_0/G_1
15	HT-1080	Osteosarcoma	G_2/M
16	A549	Lung carcinoma	$G_0/G_1+G_2/M$
17	OVCAR-8	Ovary adenocarcinoma	G_2/M
18	PC3	Prostate adenocarcinoma	G_2/M
19	U251	Glioma	G_2/M
20	RDES	Ewing’s sarcoma	G_1/S
21	HT-29	Colon adenocarcinoma	G_2/M
22	CACO-2	Colon adenocarcinoma	G_2/M
23	HCT-116	Colon adenocarcinoma	N/A[c]
24	MDA-MB2 31	Breast adenocarcinoma	S, G_2/M
25	NCI-H460	Large cell lung cancer	G_2/M

[a] Predominant type of the cell cycle arrest.

[b] Lines in which MYC-depletion causes apoptosis in more than 20% of cells at day 5 post infection.

[c] No apparent changes in the cell cycle parameters.