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The broad goals of the work supported by this grant are twofold. The first is to develop and deploy a facile system for complementation screening in cultured mammalian cells. The second is to understand elements of cellular mortality control. With regard to the first, we have developed and deployed the MaRX system. MaRX is a retroviral vector that is specifically designed to facilitate the delivery and recovery of complex gene libraries. Thus far, we have used this system to identify genes that regulate telomerase, to elucidate potential mechanisms of TGF-b-resistance in human breast tumors, to isolate genes that confer resistance to oncogene-induced apoptosis, to find cDNAs that allow bypass of p53-induced growth arrest and to address the reversibility of immortalization processes in murine and human cells. With regard to the second broad goal, we have demonstrated that an oncogene, c-myc, can regulate telomerase activity in normal human cells. Efforts over the past year have been aimed at understanding the relevance of telomerase in the immortalization of multiple cell types, at understanding the relationship between myc and telomerase activity in multiple cell types and at developing model systems in which the role of myc in cellular transformation can be addressed.

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(5) Introduction

The broad goals of the work supported by this grant are twofold. The first is to develop and deploy a facile system for complementation screening in cultured mammalian cells. The second is to utilize that system to understand elements of cellular mortality control. With regard to the first goal, we have developed and extensively deployed the MaRX system. MaRX is a retroviral vector that is specifically designed to facilitate the delivery and recovery of complex gene libraries from mammalian cells. Thus far, we have used this system to identify genes that regulate telomerase, to elucidate potential mechanisms of TGF-β-resistance in human breast tumors, to isolate genes that confer resistance to oncogene-induced apoptosis, to find cDNAs that allow bypass of p53-induced growth arrest and to address the reversibility of immortalization processes in murine and human cells. With regard to the second broad goal, we have demonstrated that an oncogene, c-myc, can regulate telomerase activity in normal human cells. Efforts over the past year have been aimed at understanding the relevance of telomerase in the immortalization of multiple cell types, at understanding the relationship between myc and telomerase activity in multiple cell types and at developing model systems in which the role of myc in cellular transformation can be addressed.

(6) Body

Technical Objective 1

In summary, the first technical objective was to develop and characterize a system of retroviral gene transfer reagents including vectors and packaging cells that were designed to facilitate complementation screening in mammalian cells. Progress toward this task was described in previous reports. A manuscript that summarizes the overall system appeared this year in *Science* and is attached as Appendix 1. The creation and deployment of this system comprises tasks 1-3.

Additional applications of this system have also been reported in the literature this year. Principally, a screen for genes that confer resistance to TGF-b was published in *Science* (appendix 2). This screen suggested that increased expression of MDM-2 may contribute to TGF-β resistance in human breast cancer. We also identified the transcription factor, *Twist*, as an antagonist of oncogene-dependent apoptosis (Appendix 3). We found that Twist could suppress p53 activity possibly by interfering with the ability of oncogenes to induce the expression of ARF, an upstream regulator of p53. The possibility that *Twist* is an oncogene is raised by the finding of Twist overexpression in rhabdomyosarcomas (~50%). Although this may not be directly relevant to breast cancer, these findings do demonstrate the generally applicability of the MaRX system for investigation a variety of processes that are potentially relevant to tumorigenesis.

Technical Objectives 2 and 3

Task 4. Completed during previous reporting periods.

Task 5. Screening of cDNA libraries for genes that induce telomerase.

The original goal of this application was the use of complementation screening to identify cDNAs that activate telomerase in normal human mammary epithelial cells. Toward this end, we used a MaRX cDNA library from HT1080 cells to transfer pools of 1000 cDNAs into populations of HMEC. 300 individual pools were used for infection of early passage HMEC cells. Using a TRAP assay the presence of telomerase in each of these cell populations was tested. Several populations showed weak activity and the corresponding cDNA pools were each subdivided into 20 pools of 100 clones for re-testing. In no case did the subdivided pools induce telomerase in naïve HMEC. Therefore, we have thus far failed to identify by a non-biased approach a gene that can regulate telomerase. During the course of these studies, we found that a cellular oncogene, c-myc, could induce telomerase in both normal human fibroblasts and in normal human mammary epithelial cells. We have therefore halted the screening approach and have decided to concentrate on deciphering the role of myc in telomerase regulation and to address the relevance of the link between myc and telomerase in human tumorigenesis.

Task 6. Screening for M1 bypass

Over the past several years, it has become clear that arrest of cells at the M1 point is triggered by telomere depletion. This mortality control point can be overcome by activation of telomerase (Bodnar et al., 1998; Wang et al., 1998) or by inactivation of the p53 and pRb tumor suppressor pathways. We have demonstrated that expression of either hTERT or c-myc can bypass M1 and can immortalize primary human mammary epithelial cells (Wang et al., 1998). However, we have also taken an alternative approach to identify genes that could antagonize p53 activity. In collaboration with Jim Hudson and David Beach of the ICH in London, we engineered p53-null mouse embryonic fibroblast (MEF) with an inducible p53 allele. These cells which reversibly arrest upon p53 activation were used in a search for genes that could bypass p53-dependent growth arrest. A number of genes were identified among which was a cytokine, mouse macrophage migration inhibitory factor (MIF). MIF expression could bypass p53-dependent growth arrest, antagonize p53-dependent apoptosis and extend the lifespan of primary MEF. Interestingly, addition of MIF protein to the culture media had the same effects. This indicates that p53 activity and cellular lifespan can be controlled by exogenous, soluble factors. Interestingly, MIF accumulates to extremely high levels at sites of chronic inflammation. There has been a longstanding but mysterious correlation between chronic inflammation and tumor formation. One intriguing possibility that arises from our work is that cytokines present at sites of inflammation may protect normal cells from apoptosis through inactivation of the p53 damage-response pathway; however, chronic suppression of p53 through these same mechanisms may contribute to cancer at chronic inflammation sites. This work is described and discussed in

more detail in a manuscript that has been accepted for publication in J. Exp. Med. (Appendix 4).

Task 7. – discussed above (task 5)
Task 8. – discussed above (task 6)

Task 9 – Investigation of the biological function of genes that are able to induce telomerase activity.

As described above, we have found that myc could induce telomerase activity. This is intriguing since myc is a human oncogene that is activated in wide spectrum of human cancers. During this granting period, we have taken a number of approaches to understand that relevance of the link between myc and telomerase. First, we have investigated the ability of telomerase and myc to immortalize primary human epithelial cells. Second, we have begun to characterize the cells which have been immortalized by telomerase activation. Third, we have begun to build model systems in which the roles of myc in cellular transformation can be investigated.

Single step immortalization of human keratinocytes

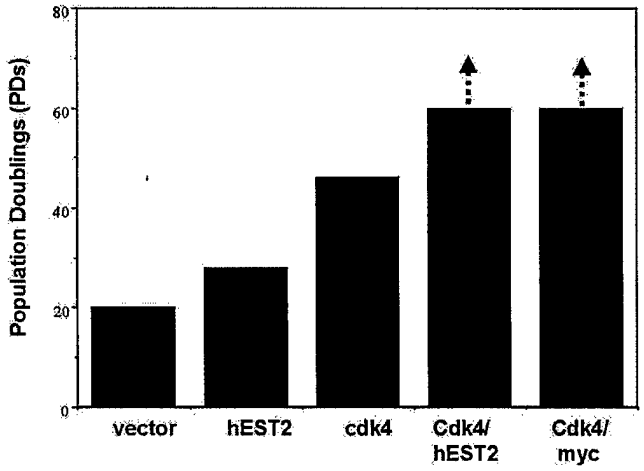
We have tested the ability of myc to regulate telomerase in normal human keratinocytes and in normal human endothelial cells (not shown). We have also investigated the ability of myc and telomerase to participate in the immortalization of these cell types.

In culture, primary human keratinocytes undergo a limited number (~20) of population doublings (PD) before encountering the M0 block and entering senescence. Although primary human mammary epithelial cells (HMECs), are also limited by an M0 block, they show a high rate of spontaneous escape the correlates with inactivation of p16 INK4a. We have therefore chosen these cells as a model for our studies.

M0 control is associated with activation of the p16INK4a/Rb growth control pathway. In primary keratinocytes and in primary HMECs, M0 can be bypassed by expression of a viral oncoprotein, HPV-16 E7 (Foster and Galloway, 1996; Kiyono et al., 1998). However, E7 is not an ideal candidate for a component of a reversible immortalization strategy. E7 is a multifunctional protein that binds to all members of the Rb family, pRb, p107, and p130. E7 also complements mutants of E1A that are defective in p300 binding (Wong and Ziff, 1996). Finally, E7 abrogates p53-dependent growth arrest (Demers et al., 1994), and loss of p53 leads to genomic instability. To minimize the oncogenic potential of our M0 bypass strategy and to limit effects on the recipient cells, we have used a more directed approach.

The p16 protein suppresses cell proliferation though stoichiometric inhibition of CDK4 and CDK6 kinases (reviewed in Sherr, 1996). Sites of interaction between these proteins have been mapped. Mutation of critical

resides in CDK4/6 can prevent the binding of INK proteins and create a kinase that resists inhibition (e.g, CDK4-R24C; Wolfel et al., 1996). As a potential M0 bypass strategy, we have introduced into human keratinocytes, expression constructs that direct the expression of either wild-type CDK4 or a CDK4 mutant that is p16 resistant. Cells infected with a control virus (e.g. empty vector or a β-galactosidase virus) senesce after 4-5 passages in culture (~20 pd). However, cells that express either the wild-type or the mutant CDK4 alone acquire an extended lifespan that was not afforded by activation of telomerase (Fig 1). CDK4-expressing cells proliferate for an additional ~30-40 PD at which point they also senesce, presumably because they have reached M1. Thus, expression of CDK4 and consequent titration of p16 INK4a was sufficient for M0 bypass and extension of lifespan. Cell in which M0 has been overcome could be immortalized by expression of either myc or hTERT, neither of which is sufficient for immortalization on its own.



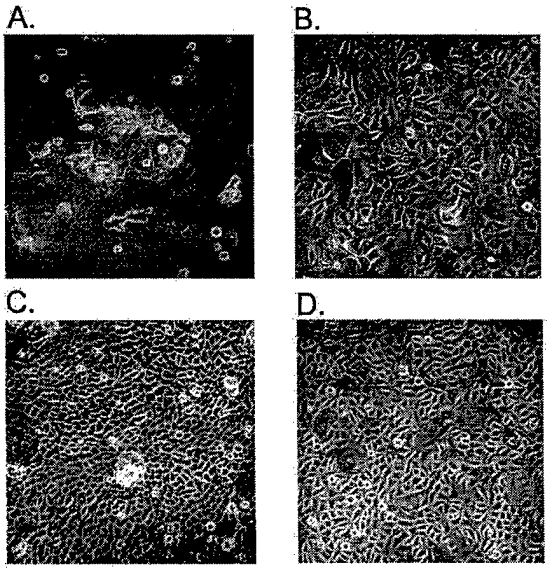


Fig 1. Extension of keratinocyte lifespan. left. Lifespan of primary human keratinocytes that have been engineered to express either single gene or binary gene combinations. The arrows indicate that

populations are continuing to proliferate. B. Photographs of engineered cells as indicated. Right. Engineered keratinocyte populations. A. Vector-infected cells at p6. B. CDK4-expressing cells at p10. C. CDK4/myc cells at p15. D. CDK4/hEST2 cells at p15.

Characterization of extended lifespan cells

It has recently been reported that hTERT expression can immortalize some normal human cells in a single step without a selection for any oncogenic mutations (Morales et al., 1999,Jiang et al., 1999). This conclusion was drawn from a lack of observed effect on the karyotype of immortal cells and the maintained integrity of some tumor suppressor pathways. We have carried out an equally superficial analysis of our immortalized cell populations. However, our results lead to somewhat different conclusions.

As a first step toward determining whether immortalization with hTERT or c-myc selects for additional alterations we examined the expression of p16, p53 and p21 at several intervals during the continuous passage of extended lifespan cultures. In accord with published results, p53 remained unchanged, and PCR-SSCP analysis of the p53 gene indicated a lack of mutation. The expression of p21 diminished as extended-lifespan cells proliferate in culture; however, the root of this phenomenon remains to be established. In HMEC the p16 protein is not detectably expressed. In these cells, the p16 locus is silenced during escape from M0, the early, telomere-independent mortality control point (Foster et al., 1998). In fibroblasts, p16 accumulates as extended lifespan populations pass the normal senescence point. However, upon further propagation, the expression of p16 is extinguished. We are still determining the basis of this phenomenon. However, loss of p16 expression can certainly be considered an oncogenic event.

Previous studies have demonstrated that hTERT will not cooperate with certain viral oncoproteins to transform primary human cells. We asked a simpler question. We sought to determine whether cells that had been immortalized by hTERT expression became mortal again upon removal of the exogenous gene. This was accomplished by delivery of hTERT on a retroviral vector that could be removed from the genome through the action of a site-specific recombinase (see Aim 3 for a description of this vector system). hTERT-expressing HMEC were propagated for ~70 population doublings beyond their normal senescence point after which the exogenous hTERT gene was removed. Our expectation was that telomerase would be silenced and that these cells would display a finite lifespan.

To our surprise, single cell clones that, according to Southern blots, had lost the hTERT expression construct retained considerable telomerase actvity (Fig 2). Furthermore, these cells have been maintained subsequently in culture for more than 8 months, and therefore, may possess an unlimited lifespan. In an attempt to determine the mechanism that sustained telomerase activity, we examined the only known regulator of the enzyme. Each of the clones from which the hTERT virus had been expunged displayed increased levels of myc expression (Fig 2). These approximated the levels of myc that were achieved

upon ectopic myc expression and that had proven sufficient to immortalize primary human cells. Similarly increased myc levels were detected in the bulk populations of hTERT-immortalized cells, ruling out the possibility that elevated myc levels arose during isolation of clonal, hTERT-excised cell lines (Fig 2).

Based upon these results, we conclude that in at least some cell types, immortalization by hTERT is accompanied by selection for additional oncogenic mutations. Furthermore, hTERT will not immortalize some primary epithelial cells without prior inactivation of the Rb/p16 tumor suppressor pathway (Kiyono et al., 1998, and our unpublished results). Thus, the therapeutic use of hTERT-expressing cells should approached with considerable caution.

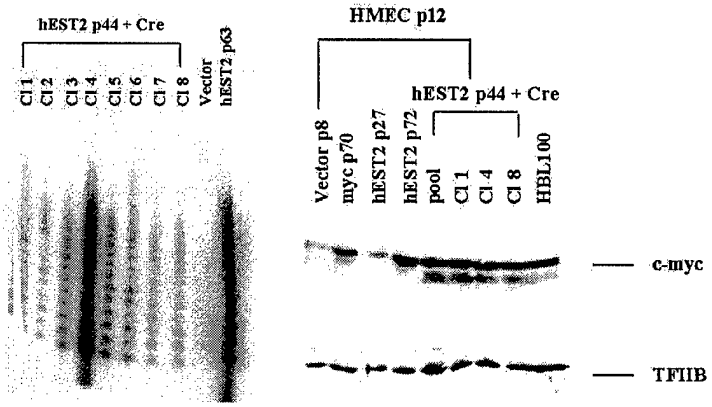


Fig. 2 **left**. Telomerase activity of single-cell HMEC clones from which the hTERT virus has been removed. For comparison, vector and hTERT (hEST2) infected cells are shown. Activity is significantly higher in C1.4 because this clone retained the hTERT construct. **right**. Analysis of c-myc protein expression in HMEC populations. Single cell clones (C1.1, C1.4 and C1.8) are analyzed in comparison to early passage vector infected cells (vector), early and late passage pools of HMEC/hTERT and an immortal breast cell lines (HBL100). TFIIB serves as a loading control

Model systems to decipher the role of myc in tumorigensis

In a recent publication, the Weinberg lab has described the transformation of normal human cells with defined genetic elements (Hahn et al., 1999). These were telomerase (hTERT), SV40 large T antigen and Ha-rasV12. This combination was sufficient to promote anchorage-independent growth of normal fibroblasts and epithelial cells and to allow tumor formation in athymic mice. Since myc can activate telomerase, we have begun to pursue the possibility that myc might substitute for hTERT in this experimental system. The ultimate goal of this line of investigation would be to reveal situations in which myc but not hTERT could promote transformation. This might shed light on the still mysterious mechanisms through which myc contributes to the development of human tumors.

As a starting point, we have introduced into normal human fibroblasts, myc or hTERT in combination with viral oncoproteins (SV40 T antigen, HPV E6

and E7 proteins) and ras. Cells are selected for the presence of the three components and plated in soft agar to assess anchorage independent growth.

We felt that the first step was to reproduce the previously published results. We therefore combined myc or hTERT with T and rasV12. Unfortunately, we were unable to obtain any viable cells that express the combination of these oncogenes. It is well established that activated ras can induce senescence (Serrano et al., 1997), and the morphology of our ras-infected population is characteristic of senescent cells. We therefore entertained the possibility that the dominance of the pro-senescence effects of ras resulted from supplying too much flux through ras effector pathways. We therefore repeated this set of experiments using mutants of rasV12 that are compromised in specific effector pathways. We have been able to obtain anchorage independent growth with cells that express myc or hTERT in combination with T antigen and an effector mutant of ras that preferentially activates the raf/map kinase pathway (Table 1.). Interestingly a much stronger response was seen with myc in combination with E6/E7 and the same effector loop mutant. In this case, transformation was seen only with myc and not with hTERT, although both activate telomerase and immortalize the target cell population. This reveals one possible situation in which this developing model system can be used to reveal roles of myc in human tumorigenesis that extend beyond telomerase activation.

Table 1. transformation of human BJ fibroblasts– soft agar assay

Immortalizing gene	Viral oncogene	Ras	Soft agar colonies
-	-	-	-
myc	T antigen	HrasV12	- (senesced)
myc	T antigen	HrasV12S35	++
myc	E6/E7	HrasV12	-(senesced)
myc	E6/E7	HrasV12S35	++++
hTERT	T antigen	HrasV12	- (senesced)
hTERT	T antigen	HrasV12S35	++
hTERT	E6/E7	HrasV12	- (senesced)
hTERT	E6/E7	HrasV12S35	- (not senescent)

We do not yet understand our failure to reproduce precisely the Weinberg result. We have not yet investigated the effects of order of addition of the transforming genes to the target cell populations. I do not intend to present these results as a comprehensive story that we fully understand. I merely wish it illustrate preliminary progress as an indication of the direction that we will take over the next year in our efforts to investigate the role of myc in human tumor formation.

Task 10 – see task 8 and appendix 4

Task 11 – see task 8 and appendix 4

(7) Key Research Accomplishements

- myc activates telomerase in a variety of normal human cell types
- myc can immortalize normal human cells – alone or in combination with inactivation of the p16/Rb pathway
- myc can transform normal human cells upon combination with other oncogenes
- MIF can antagonize p53 activity and extend the lifespan of MEF

(8) Reportable outcomes (4 manuscripts)

Hannon, G. J., Sun, P., Carnero, A., Xie, L.-Y., Maestro, R., Conklin, D. and Beach, D. (1999) MaRX: An approach to Genetics in mammalian cells. Science 283:1129-1130. Appendix 1.

Sun, P., Dong, P., Dai, K., Hannon, G. J. and Beach D. (1998) p53-independent role of MDM2 in TGF-b1 resistance. Science 282:2270-2272. Appendix 2.

Maestro, R., Dei Tos, A. P., Hammamori, Y., Krasnokutsky, S., Sartorelli, V., Kedes, L., Doglioni, C., Beach, D. and Hannon, G. J. (1999) twist is a potential oncogene that inhibits apoptosis. Genes & Dev. 13:2207-2217. Appendix 3.

Hudson, J. D., Shoaibi, M. A., Maestro, R., Carnero, A., Hannon, G. J. and Beach D. (1999) J. Exp. Med., in press. Appendix 4.

(9) Conclusions

Accumulating evidence suggests that telomerase activation is a key component of human tumorigenesis. Repression of telomerase can inhibit the growth of human tumor cells in culture (Zhang et al., 1999; Hahn et al., 1999) and activation of telomerase – and consequent cellular immortalization -- is essential for transformation of normal human cells in vitro. We have provided a link between a known oncogene, c-myc, and telomerase. This provides one possible component of the role of c-myc in human cancer. Though the support of this grant, we have begun to build model systems that may allow us to decipher roles of myc in promoting tumorigenesis that extend beyond its ability to immortalize. Ultimately this work may contribute to understanding the cellular pathways that are commonly altered in human cancer.

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(11) Appendices

Hannon, G. J., Sun, P., Carnero, A., Xie, L.-Y., Maestro, R., Conklin, D. and Beach, D. (1999) MaRX: An approach to Genetics in mammalian cells. Science 283:1129-1130. Appendix 1.

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SCIENCE

Appendix 1

MaRX: An Approach to Genetics in Mammalian Cells

Gregory J. Hannon, Peiqing Sun, Amancio Carnero, Lin Ying Xie, Roberta Maestro, Douglas S. Conklin, David Beach*

MaRX: An Approach to Genetics in Mammalian Cells

Gregory J. Hannon, Peiqing Sun, Amancio Carnero, Lin Ying Xie, Roberta Maestro, Douglas S. Conklin, David Beach*

A genetic approach is the most direct way to elucidate biological processes that are poorly understood. One of the first such efforts—the landmark study of Beadle and Tatum (*1*) on the genetics of metabolic pathways—established the influential "one gene, one enzyme" hypothesis. In subsequent decades, the yeasts *Saccharomyces cerevisiae* and *S. pombe* became the premier genetic models. The oft-touted "power of yeast genetics" was not fully realized, however, until classical techniques were combined with an ability to manipulate the organisms with recombinant DNA methods (*2–4*). Thus were conceived the tools that today make yeasts the best-characterized eukaryotes. These tools, however, have limitations: accumulating human sequence data reveals many genes that are not represented in yeast. How can the leap be made from yeast to human?

To solve this problem, we sought to apply genetic methods to mammals or their manipulable surrogates, cultured mammalian cells. Rather than creating a genetic methodology that technically mirrors the approach in yeast, we developed one with comparable genetic access to mammalian biology—the MaRX system.

To date, the application of molecular genetics to cultured mammalian cells—probably the most widely used model "organisms" in mammalian biology—has proved problematic. Only sporadic attempts have been made (*5–10*), despite the fact that many disease-related processes can be readily investigated in tissue culture. Because they are asexual diploids, mammalian cells are inaccessible to all classical genetic methods except mutagenesis.

A variety of technical barriers impede the use of genetic cloning approaches in mammalian cells. First, the inability to perform genetic crosses prevents the creation of complex mutant cell strains. Second, mammalian cells in culture are more plastic than are free-living microorganisms. Thus, significant variation occurs even among cells derived from a clonal isolate. Ultimately, this translates into phenotypes that are more prone to spontaneous reversion than those used for genetic selection in microorganisms. Finally, the vectors currently available for genetic manipulation of cultured cells are relatively primitive.

We therefore developed the MaRX system, a specialized strategy to facilitate function-based gene isolation in mammalian cells. This system relies on two concepts to overcome impediments to the use of genetic methods in cultured cells. First is the use of nucleic acid as a "virtual mutagen" rather than reliance on chemical or other mutagens. Cloned complementary DNAs (cDNAs), either in the sense or antisense orientation, are used to reversibly alter gene expression thereby creating a phenotype in a cultured cell. Second, the tendency of such phenotypes to revert spontaneously has been accommodated. The system allows efficient introduction of cDNA libraries into target cells, and allows efficient recovery of either individual genes or complex sublibraries from cell populations that have been enriched on the basis of a specific biological characteristic. As shown in Fig. 1, the essence of the approach is the ability to rapidly filter complex mixtures of clones through multiple rounds of phenotypic selection, termed cycle cloning.

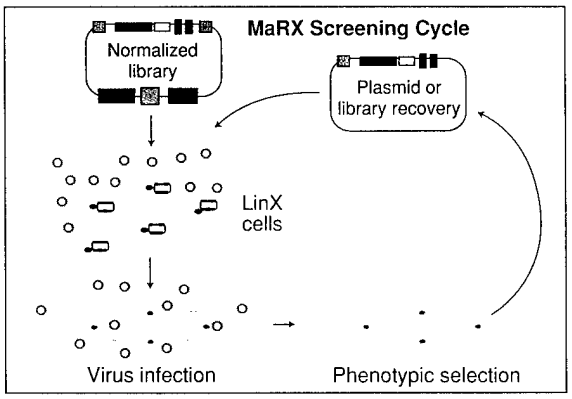

Fig. 1. The MaRX cycle. A normalized DNA library is converted into a library of retroviruses by using a packaging cell line (linX). Then, these infected recipient cells are selected or enriched on the basis of a specific biological property. Proviruses are recovered and used for virus production and subsequent rounds of screening.

The difficulty of manipulating large numbers of tissue culture cells coupled with the need to screen complex libraries dictates the need for efficient gene transfer. To achieve this goal in a wide range of cell types, we relied on newly designed, replication-deficient retrovirus vectors. The genomic structure and replication of these viruses is well understood, thus simplifying modification of existing systems for use as genetic tools. Furthermore, stable integration of recombinant retroviruses allows phenotypes to be assessed over many cell generations. However, downstream analysis of cDNAs that elicit selected phenotypes is complicated by the need to recover a single-copy provirus from the host genome. Previous applications of the retroviral vectors for functional cloning have relied on polymerase chain reaction (PCR) for isolation of virus-borne cDNA fragments (*6*, *7*, *10*). This approach may be sufficient when only a few cell clones need to be analyzed. However, PCR-based approaches are ill suited for manipulation of complex populations.

We addressed the problem of efficiently recovering integrated retroviruses by incorporating into MaRX the ability to be excised from genomic DNA by the action of a site-specific recombinase either in vitro or in vivo (Fig. 2A). The effect of excision in vivo is loss of the integrated virus. This provides a simple mechanism to demonstrate that the phenotype of a selected cell requires expression of the exogenous genetic element—a reversion test (Fig. 2B). Excision in vitro is accomplished by treating purified genomic DNA with the appropriate recombinase. This generates a circular molecule carrying the sequence responsible for generating the desired phenotype. To facilitate recovery of this excised virus, we have included within the MaRX provirus an optimized mini-plasmid (~700 base pairs in length). Thus, the excised provirus can be rescued simply by transforming recombinase-treated genomic DNA into highly competent *Escherichia coli*. This excision protocol also allows recovery of individual genes or complex mixtures of proviruses.

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The excised MaRX provirus has a single intact long-term repeat sequence. Upon transfection into an appropriate packaging cell line, the recovered provirus yields infectious retrovirus with an efficiency similar to that of the intact MaRX vector. Therefore, verification of a genetic rescue can be achieved without manipulation of individual candidate fragments. The ability to rescue a library of functional proviruses from a selected cell population allows a gene to be enriched from complex mixture through multiple rounds of phenotypic selection (through use of a single cell line; multiple, different cell lines; or selection criteria). Because many interesting phenotypes are "leaky," the ability to pass complex populations through multiple rounds of selection (cycle cloning) allows access to a wider range of biological problems.

The primary motivation for creating the MaRX system was to enable the cloning of mammalian genes by relying solely on their functional properties. To test the efficacy of our approach, we sought to reproduce one of the first marker rescue experiments to succeed in cultured cells, the cloning of the *ras* oncogene (*8*).

NIH-3T3 cells were infected with a MaRX cDNA library derived from a tumor cell line. A screen for transformation yielded a number of foci. Many of the isolates that displayed the most highly transformed phenotype carried MaRX proviruses encoding activated *ras* alleles. In a screen that took only 8 weeks, *ras* was isolated four independent times from 20 standard (100 mm) tissue culture plates. Thus, this simple model verified our ability to isolate relevant genes through phenotypic selection.

The ability to probe the function of specific genes through the creation of loss-of-function "alleles" is at the heart of any genetic methodology. The diploid nature of mammalian cells necessitates the use of unconventional approaches to the creation of "recessive" mutants. Effective inhibition of gene function can occur following expression of antisense RNAs (*11*). We used this method to test the MaRX system's ability to assess the consequences of loss of the tumor suppressor gene *p53*.

We created a directional, randomly primed cDNA library consisting of fragments of the *p53* coding sequence, because antisense mRNA fragments may inhibit gene expression more effectively than complete antisense mRNAs (*10*). This library was transferred into A3 cells, a murine embryonic fibroblast (MEF) derivative that ectopically expresses a conditional version of *p53* from a strong viral promoter. Shift of these cells to the permissive temperature resulted in cell-cycle arrest. Infection of A3 cells with the antisense *p53* mini-library allowed colony formation at a frequency of roughly 10^{-3} per cell.

Proviruses containing interfering *p53* fragments were recovered from clones that resisted growth arrest. Expression of the most highly represented fragments resulted in a >90% inhibition of p53 protein expression and in efficient rescue of growth arrest. In primary cells (MEF cells), the selected fragments inhibited expression of endogenous p53, extended life-span, and protected the cell from DNA-damaging agents. Excision of the p53-inhibitory provirus (the "virtual mutagen") from any of these cell populations reverted the p53-null phenotype, demonstrating a continuous dependence on the antisense RNA. Expression of a full-length *p53* antisense RNA produced effects that were indistinguishable from those seen with the selected fragments. Thus, at least in this instance, use of a restricted antisense gene fragment was not required to generate a phenotype.

These results demonstrate that the MaRX system can create reversible, loss-of-function phenotypes. Antisense RNAs can work effectively against endogenous mRNAs and can even inhibit expression from ectopically expressed transcripts.

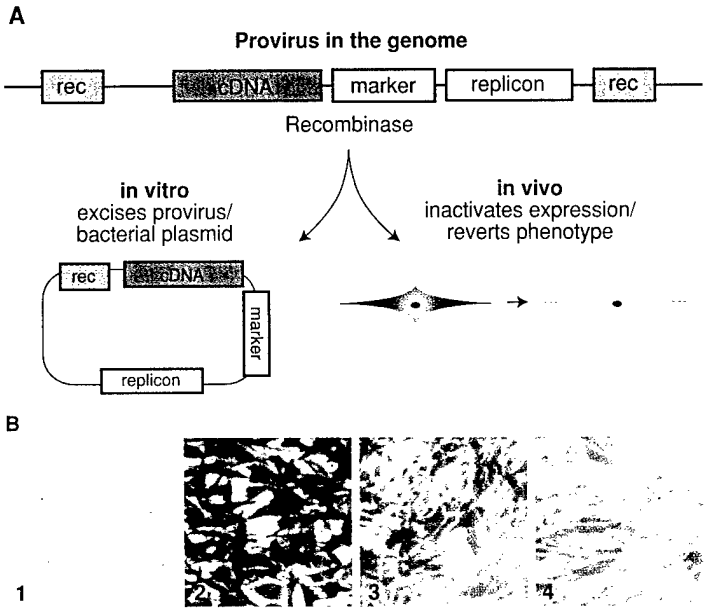

Fig. 2. Recombinase excision of the MaRX vector. (**A**) A representation of the integrated MaRX provirus and the consequences of recombinase treatment in vitro and in vivo. (**B**) NIH-3T3 cells (1) were infected with a MaRX virus that directs β-galactosidase expression (2). These cells were then transduced with a retrovirus that directs recombinase expression (3). Upon recombinase expression and continued passage (4), the phenotype of these cells (β-galactosidase expression) is reverted.

We have further validated the MaRX system in a range of biological contexts. For example, we investigated the roles of tumor suppressor function in cellular senescence, studying multiple genes and their interactions, and of inhibitory cytokines in growth control (*12, 13*). A search for genes that protect from oncogene-dependent cell death revealed a potential oncogene (*14*), and a screen for bypass of *p53* function uncovered a possible explanation for the long-mysterious link between chronic inflammation and cancer (*15*). We even deployed the MaRX system to identify genes that confer resistance to widely used pharmaceuticals (*16*). The versatility of the approach is exemplified by adaptation of the MaRX system to create two different versions of a high-throughput, homologous, mammalian secretion trapping system that is capable of identifying type I, type II, and unconventional secretion signals (*17*).

We have created a coherent system of reagents that enables a powerful genetic screening approach to a broad range of biological problems in mammalian cells. In principle, this system can be used to investigate any aspect of biology or pathobiology that can be recapitulated in a cell-culture model. In due course, we anticipate elaboration of MaRX into a recombinant mutagen for whole-animal studies.

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Appendix 2

p53-Independent Role of MDM2 in TGF-β1 Resistance

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p53-Independent Role of MDM2 in TGF-β1 Resistance

Peiqing Sun, Ping Dong, Kang Dai,* Gregory J. Hannon, David Beach†

Transforming growth factor β (TGF-β) inhibits cell proliferation, and acquisition of TGF-β resistance has been linked to tumorigenesis. A genetic screen was performed to identify complementary DNAs that abrogated TGF-β sensitivity in mink lung epithelial cells. Ectopic expression of murine double minute 2 rescued TGF-β–induced growth arrest in a p53-independent manner by interference with retinoblastoma susceptibility gene product (Rb)/E2F function. In human breast tumor cells, increased MDM2 expression levels correlated with TGF-β resistance. Thus, MDM2 may confer TGF-β resistance in a subset of tumors and may promote tumorigenesis by interference with two independent tumor suppressors, p53 and Rb.

The TGF-β signaling pathway has been implicated in tumor suppression (*1*). Loss of TGF-β sensitivity is frequently observed in tumors derived from cells that are normally sensitive, and the extent of TGF-β resistance often correlates with malignancy (*2*). Some tumors may develop TGF-β resistance following inactivation of essential components of the TGF-β signaling pathway (*3–5*) or through deletion of the $p15^{INK4B}$ locus (*6*). However, such alterations cannot account for the majority of cases in which TGF-β responsiveness is lost. Therefore, TGF-β resistance must also be achieved by other mechanisms.

To identify genetic alterations that lead to TGF-β resistance in tumor cells, we screened for genes that, when overexpressed, allow cells to escape TGF-β–induced growth arrest (*7*). A cDNA library was introduced into Mv1Lu, a TGF-β–sensitive mink lung epithelial cell line, using a retrovirus-based genetic screening system (*8*). Infected cells were selected for the ability to sustain proliferation in the presence of TGF-β. We recovered three genes that conferred TGF-β resistance: *Mdm2* c-*myc*, and *NF-IX-1* (Fig. 1, top panel). When treated with TGF-β, cells expressing MDM2, c-*myc*, or NF-IX-1 formed colonies and were morphologically identical to untreated cells (Fig. 1) (*9*). MDM2 also conferred TGF-β resistance in human mammary epithelial cells (HMECs) (Fig. 1, bottom panel).

The isolation of c-*myc*, a gene previously shown to overcome TGF-β–induced arrest (*10*), validated the genetic screen. NF-IX-1 is a member of a family of transcription factors that may function in development and differentiation (*11*). The mechanism by which NF-IX-1 confers TGF-β resistance remains to be investigated. Because MDM2 is an oncogenic protein that is commonly overexpressed in a broad spectrum of tumors (*12*), we focused on understanding how this protein confers TGF-β resistance.

Activation of TGF-β signaling regulates the expression of a battery of genes. MDM2 overexpression in Mv1Lu cells did not alter the response of known TGF-β targets (for example, *PAI-1*, *p15*, c-*myc*, and *cdc25A*) (*9*), indicating that MDM2 does not confer resistance by disruption of TGF-β signaling.

MDM2 associates with and inactivates the tumor suppressor protein, p53. To test the possibility that MDM2 bypasses TGF-β–induced growth arrest through an effect on p53, we investigated whether interference with p53 activity could produce cytokine resistance. Two dominant-negative p53 alleles, p53Val135 (a temperature-sensitive mutant) or p53-175H (*13–15*) were introduced into Mv1Lu cells, which contain endogenous, wild-type p53 (*16*). The functionality of these p53-interfering mutants was confirmed by their ability to suppress p53-dependent transcription (*9*). Cells in which p53 had been inactivated by

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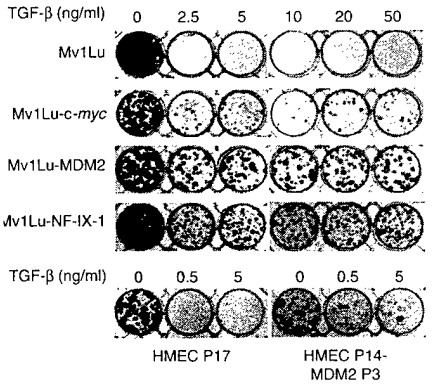

Fig. 1. *Mdm2*, c-*myc*, and *NF-IX-1* bypass TGF-β–induced growth arrest. (Top panel) Control Mv1Lu or Mv1Lu expressing c-*myc*, MDM2, or NF-IX-1 (4000 cells) were treated with TGF-β for 8 days. (Bottom panel) HMECs at passage 14 were infected with a retroviral vector that drives MDM2 expression, and infected cells were selected with hygromycin. After three more passages, HMECs expressing MDM2 or control HMECs (4000 cells) at passage 17 were treated with TGF-β for 16 days. All cells were visualized by staining with crystal violet.

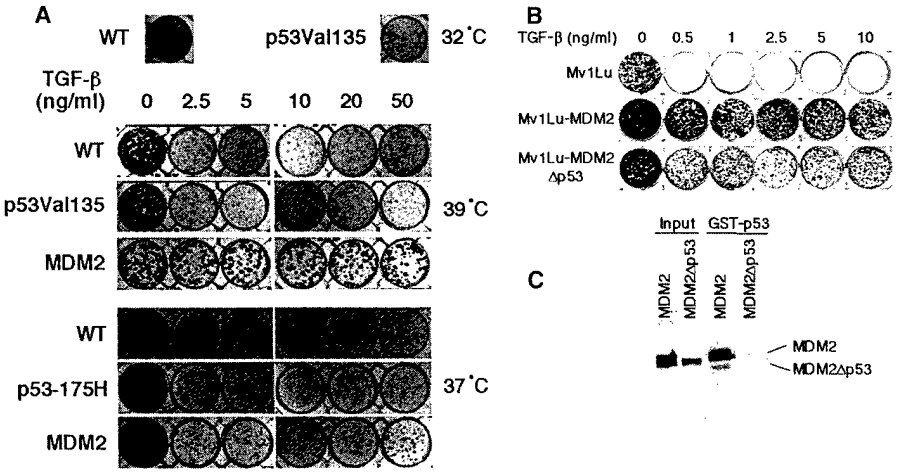

Fig. 2. MDM2 confers TGF-β resistance through a p53-independent mechanism in Mv1Lu cells. (**A**) Control Mv1Lu cells or cells expressing MDM2, p53Val135, or p53-175H were treated with TGF-β for 8 days. (**B**) Control Mv1Lu cells or cells expressing MDM2 or an MDM2 mutant that cannot bind p53 were treated with TGF-β for 8 days. (**C**) Wild-type or mutant MDM2 proteins were translated in vitro from pcDNA3 in the presence of [^{35}S]methionine and were incubated with a glutathione *S*-transferase (GST)–p53 fusion protein bound to glutathione–Sepharose 4B beads. Proteins that remained bound to beads after washing (right two lanes) were separated by 12% SDS–polyacrylamide gel electrophoresis, and radiolabeled proteins were visualized by autoradiography. A portion of each in vitro translation reaction is shown for comparison (left two lanes).

expression of either dominant-negative mutant retained TGF-β sensitivity (Fig. 2A). Furthermore, an MDM2 mutant from which the p53-binding domain had been removed failed to bind p53 (Fig. 2C) but still conferred TGF-β resistance (Fig. 2B). Thus, MDM2 overcomes TGF-β through a mechanism that is distinct from its ability to inactivate p53.

TGF-β induces G_1 arrest through effects on the Rb/E2F pathway (*17–19*). Because expression of human papillomavirus HPV-16 E7 protein, which abolishes Rb but not p53 function (*20*), conferred TGF-β resistance in Mv1Lu cells (*9*), we investigated the possibility that MDM2 could bypass TGF-β by interference with the RB/E2F pathway. This hypothesis is consistent with the recent finding that MDM2 can bind directly to Rb and E2F/DP transcription factors (*21, 22*).

In control Mv1Lu cells, TGF-β treatment led to a gradual change in Rb phosphorylation status (Fig. 3A). After 24 hours (the time at which growth arrest was established), the majority of Rb had shifted from the hyperphosphorylated form to the growth-inhibitory, hypophosphorylated form. However, in MDM2- and c-*myc*–expressing cells, the majority of Rb remained in hyperphosphorylated, non–growth-inhibitory state.

E2F proteins are transcription factors that bind to unphosphorylated Rb. Rb phosphorylation releases E2F proteins in an active, growth-promoting form (*23*). The effect of MDM2 on Rb phosphorylation predicted that MDM2 would have a positive effect on E2F activity. In contrast to previous studies in other cell lines (*21, 22*), expression of MDM2 in Mv1Lu cells did not increase the activity of an E2F-dependent reporter construct (Fig. 3B). TGF-β treatment reduced transcription of this reporter by twofold. However, MDM2 expression prevented this reduction (Fig. 3B). Alteration of E2F activity by either TGF-β treatment or MDM2 overexpression reflected changes in E2F-1 protein levels (Fig. 3C). TGF-β treatment led to a gradual decrease in E2F-1, and this decrease was prevented by ectopic MDM2 expression. These results indicate that MDM2 rescues TGF-β–induced growth arrest, at least in part, through maintenance of E2F-1 protein levels and E2F activity. Similar effects were evident in cells that ectopically express c-*myc* (Fig. 3, B and C), suggesting that c-*myc* and MDM2 may bypass TGF-β–induced arrest through overlapping mechanisms.

MDM2 is frequently overexpressed in human tumors (*12*). We identified one biological consequence of MDM2 overexpression, bypass of TGF-β–induced growth arrest. TGF-β induces growth arrest in normal human lymphocytes, melanocytes, and breast epithelial cells. However, cells from human leukemia, lymphomas, melanomas, and breast carcinomas are often TGF-β resistant (*24–27*). Coincidentally, MDM2 is commonly overexpressed in these tumors (for example, in 73% of human breast carcinomas) (*28–32*). Enforced expression of MDM2 in primary HMECs converted these TGF-β–sensitive cells to a resistant phenotype (Fig. 1, bottom panel). These observations raised the possibility that increased MDM2 expression might contribute to TGF-β resistance in tumors.

Therefore, we examined the relationship between MDM2 expression levels (Fig. 4A) and TGF-β responsiveness (Fig. 4B) in seven human breast tumor cell lines. MDM2 was expressed in T-47D, ZR-75-1, and HTB20 cells at levels comparable to those observed in cells (HMEC and Mv1Lu) that had been made TGF-β–resistant by infection with MDM2 retroviral vectors. These three cell lines were completely resistant to TGF-β–induced growth arrest. The two cell lines (MCF-7 and BT549) that were most sensitive to TGF-β treatment had very low MDM2 levels, similar to those seen in TGF-β–sensitive, normal HMECs. Thus, in several tumor cell lines, increased MDM2 expression strictly correlated with the ability to escape TGF-β–induced growth inhibition. Two other breast carcinoma cell lines (HBL100 and MDA-MB-468) exhibited partial resistance to TGF-β despite low levels of MDM2 ex-

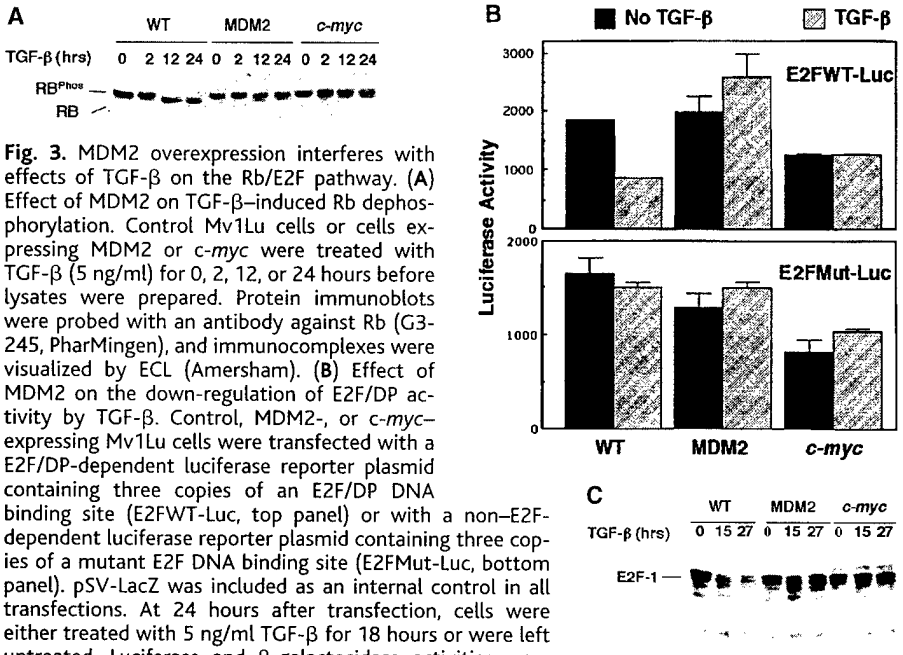

Fig. 3. MDM2 overexpression interferes with effects of TGF-β on the Rb/E2F pathway. (**A**) Effect of MDM2 on TGF-β–induced Rb dephosphorylation. Control Mv1Lu cells or cells expressing MDM2 or c-*myc* were treated with TGF-β (5 ng/ml) for 0, 2, 12, or 24 hours before lysates were prepared. Protein immunoblots were probed with an antibody against Rb (G3-245, PharMingen), and immunocomplexes were visualized by ECL (Amersham). (**B**) Effect of MDM2 on the down-regulation of E2F/DP activity by TGF-β. Control, MDM2-, or c-*myc*–expressing Mv1Lu cells were transfected with a E2F/DP-dependent luciferase reporter plasmid containing three copies of an E2F/DP DNA binding site (E2FWT-Luc, top panel) or with a non–E2F-dependent luciferase reporter plasmid containing three copies of a mutant E2F DNA binding site (E2FMut-Luc, bottom panel). pSV-LacZ was included as an internal control in all transfections. At 24 hours after transfection, cells were either treated with 5 ng/ml TGF-β for 18 hours or were left untreated. Luciferase and β-galactosidase activities were then measured (Promega). Values represent means ± SEM of luciferase activities (normalized to β-galactosidase activities) from three independent transfections. (**C**) Effect of MDM2 on the down-regulation of E2F-1. Control, MDM2- or c-*myc*–expressing Mv1Lu cells were treated with 5 ng/ml TGF-β for 0, 15, or 27 hours. Cell lysates were prepared, and protein immunoblots were probed with an antibody against E2F-1 (C20, Santa Cruz Biotechnology).

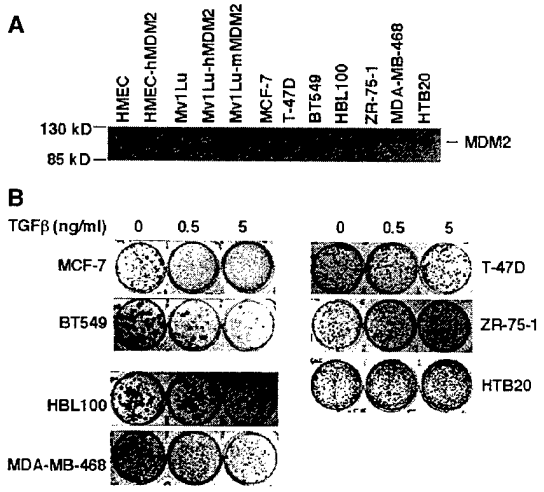

Fig. 4. MDM2 expression correlates with resistance to TGF-β in human breast tumor cell lines. (**A**) MDM2 protein levels in human breast tumor cell lines. Cell lysates were prepared from the indicated cell lines, and protein immunoblots were probed with an antibody to MDM2 (2A10). (**B**) TGF-β responsiveness of human breast tumor cell lines. Human breast tumor cell lines were treated with TGF-β at the indicated concentrations for 20 days.

pression, confirming that other mechanisms (for example, c-*myc* overexpression, receptor mutation, and so forth) must also contribute to TGF-β resistance.

As breast carcinomas and melanomas become metastatic, they secrete large amounts of TGF-β (*25, 27*). This may enhance tumor cell invasion through effects on extracellular matrix (*27, 33*). Thus, TGF-β resistance may be an essential adaptation to the metastatic phenotype. In accord with this notion, the extent of TGF-β resistance correlates with metastatic progression (*28, 30*), and targeted deletion of an essential component of the TGF-β signaling cascade, *Smad3*, promotes the formation of metastatic tumors (*1*). Although TGF-β resistance can be achieved through multiple routes, increased expression of MDM2 is sufficient to confer this phenotype.

Previous work indicated that MDM2 may contribute to transformation through mechanisms that are independent of effects on p53. For example, in some human breast carcinomas and lymphomas, p53 mutation and MDM2 overexpression occur together (*31, 32*). Recently, alternatively spliced forms of MDM2 were identified in bladder and ovarian carcinomas (*34*). These alternative forms lack the p53-binding domain but still transform NIH-3T3 cells. We have demonstrated that MDM2 can overcome growth inhibition by TGF-β through effects on the RB/E2F pathway. These results provide a potential mechanism underlying p53-independent oncogenic activities of MDM2. Thus, in tumors, MDM2 may antagonize both the Rb and p53 pathways, functioning in many respects as a cellular version of SV40 large T antigen.

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7. A library was made from Swiss 3T3 and Balb/c 3T3 cells and was cloned into a retroviral expression vector HygroMaRXII (*8*), packaged in an ecotropic virus packaging cell line LinX E (L. Y. Xie, D. Beach, G. J. Hannon, unpublished results), and used to infect Mv1Lu cells which had been engineered to express the ecotropic retrovirus receptor. We estimated that a total of 10^7 cells were infected. The infected cells were selected with hygromycin and then subjected to TGF-β treatment for 3 months. Integrated proviruses were then excised with Cre recombinase from genomic DNA isolated from plates containing resistance cells. cDNA from 38 plates of resistant cells have been recovered and sequenced thus far. Among these, seven plates contained a cDNA encoding *Mdm2*, one contained c-*myc*, and seven contained *NF-IX-1*.
8. The cDNA expression vector (HygroMaRXII; P. Sun, G. J. Hannon, D. Beach, unpublished data) was designed based on Molony murine leukemia virus (MoMLV). We included a recognition site (loxP) for Cre recombinase in a 3′ long terminal repeat (LTR) and a bacterial replicon and a bacterial selectable marker within the retroviral genome. These modifications allow easy and efficient recovery of cDNAs by Cre-mediated excision of integrated proviruses from the genome. The recovered circular plasmids contained a single LTR, and thus could be directly used to produce recombinant viruses for further studies.
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twist is a potential oncogene that inhibits apoptosis

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Oncogene activation increases susceptibility to apoptosis. Thus, tumorigenesis must depend, in part, on compensating mutations that protect from programmed cell death. A functional screen for cDNAs that could counteract the proapoptotic effects of the *myc* oncogene identified two related bHLH family members, Twist and Dermo1. Both of these proteins inhibited oncogene- and *p53*-dependent cell death. Twist expression bypassed p53-induced growth arrest. These effects correlated with an ability of Twist to interfere with activation of a p53-dependent reporter and to impair induction of *p53* target genes in response to DNA damage. An underlying explanation for this observation may be provided by the ability of Twist to reduce expression of the *ARF* tumor suppressor. Thus, Twist may affect p53 indirectly through modulation of the ARF/MDM2/p53 pathway. Consistent with a role as a potential oncoprotein, Twist expression promoted colony formation of *E1A/ras*-transformed mouse embryo fibroblasts (MEFs) in soft agar. Furthermore, Twist was inappropriately expressed in 50% of rhabdomyosarcomas, a tumor that arises from skeletal muscle precursors that fail to differentiate. Twist is known to block myogenic differentiation. Thus, Twist may play multiple roles in the formation of rhabdomyosarcomas, halting terminal differentiation, inhibiting apoptosis, and interfering with the *p53* tumor-suppressor pathway.

[*Key Words*: Twist; oncogenes; apoptosis; tumorigenesis; Dermo1]

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A defining characteristic of tumor cells is the escape from regulatory mechanisms that normally restrain cell proliferation. This is accomplished through the accumulation of multiple genetic alterations. Among these are the inactivation of key tumor suppression pathways and the activation of oncogenes (for review, see Vogelstein and Kinzler 1998).

The products of cellular oncogenes such as *ras* and *myc* are components of normal growth control pathways. These form part of the program that promotes entry into the division cycle in response to appropriate environmental cues. However, in tumor cells, the normal function of these genes is subverted to provide hyperactive proliferative signals. It is becoming increasingly clear that normal cells respond to inappropriate growth signals by activating homeostatic growth control pathways that protect multicellular organisms from tumor formation.

Constitutive activation of Ras promotes transformation of some immortalized cells. However, Ras activation in normal cells provokes cellular senescence. This irreversible growth arrest probably negates the ability of these cells to contribute to tumor formation (Serrano et al. 1997; Lin et al. 1998). In contrast, other cellular and viral oncogenes sensitize cells to undergo programmed cell death on exposure to stimuli that might normally cause a reversible growth arrest. For example, Myc induces apoptosis on removal of serum survival factors from primary cells or Rat1 fibroblasts (Evan et al. 1992; Hermeking and Eick 1994; Wagner et al. 1994). Similarly, primary mouse embryo fibroblasts (MEFs) that express E1A are sensitized to programmed cell death in response to contact inhibition, growth factor withdrawal, and DNA damage (Debbas and White 1993; Lowe and Ruley 1993; Lowe et al. 1993, 1994).

The growth inhibitory properties of many oncogenes dictate that tumorigenesis requires the cooperation of different classes of genes. For example, transformation by *Ras* can proceed only in the presence of additional mutations that prevent Ras-induced senescence (Serrano et al. 1997). Furthermore, the ability of Myc or E1A to promote tumorigenesis requires that transformed cells be protected from the proapoptotic effects of these oncoproteins. An understanding of oncogene cooperation requires both a knowledge of the mechanisms by which

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oncogene activation provokes homeostatic responses and a clarification of the routes through which cooperating oncogenic events defeat these protective controls.

It is clear that oncogene-transformed cells require additional genetic alterations that render them resistant to apoptotic stimuli. Such genetic changes are likely to be obligate for oncogenesis. However, the full spectrum of these events has proven difficult to elucidate, because protective genes are not likely to be discovered via the cellular transformation assays that have proven a rich source of new oncogenes. For example, *bcl-2* synergizes with *myc* in the generation of Burkitt lymphoma but does not cooperate with *myc* in transformation of mouse fibroblasts in vitro (Vaux et al. 1988). As an approach to the discovery of potential oncogenes that might elude conventional methods, we have undertaken a search for cellular genes that can counter the proapoptotic effects of *myc* activation.

Results and Discussion

A genetic screen for antiapoptotic proteins

Direct, functional selection of genetic alterations that evoke specific phenotypes has provided a powerful method for the dissection of numerous biological pathways in genetically tractable eukaryotes such as yeasts, *Caenorhabditis elegans*, and *Drosophila*. These approaches have been extended to cultured mammalian cells by several groups (e.g., Deiss and Kimchi 1991; Gudkov et al. 1994; Rayner and Gonda 1994; Wong et al. 1994; for review, see Gudkov and Roninson 1997; Kimchi 1998). Building on these prior studies, we have developed a suite of tools that streamlines the process of complementation screening in mammalian cells (Sun et al. 1998; Hannon et al. 1999).

We have designed a series of modified, replication-deficient retrovirus vectors (MaRX) and packaging cell lines (LinX) that allow high-efficiency gene transfer to a wide range of cell types. Recovery of integrated MaRX proviruses from selected cell populations is facilitated by two key modifications. First, the MaRX provirus contains, within the LTR, target sequences for a site-specific recombinase. Second, the MaRX provirus contains an optimized bacterial replicon. Treatment of genomic DNA from infected cells with the appropriate recombinase enzyme results in excision of a circular plasmid comprising the integrated virus that can be propagated in bacterial cells. Despite the fact that this plasmid contains only a single LTR, it is capable of producing infectious retrovirus on transfection into the LinX packaging cells. Thus, by this approach we can recover cDNAs that confer a specific phenotype and transfer them directly into new recipient cells without intervening cloning steps.

The *myc* oncogene can predispose a wide variety of cell types to programmed cell death. However, the design of a genetic selection for cDNAs that can counter the proapoptotic effects of *myc* requires the use of cells that die with high efficiency. Rat1/MycER cells undergo apoptosis on simultaneous Myc activation and growth factor withdrawal (Evan et al. 1992). However, even on delivery of a strong proapoptotic stimulus, a significant number of cells survive (~0.1%–1% of the population). For this reason, we could not select directly for cells carrying protective cDNAs through a single round of treatment. Instead, we designed a genetic screen in which cell populations would be exposed to multiple, iterative rounds of killing and rescue with the hope that protective cDNAs would be continuously enriched, whereas neutral cDNAs would be counter selected (Fig. 1).

To test our approach, we reconstructed the screen using a well-characterized antiapoptotic gene, *bcl-2*. Rat1/MycER cells were coinfected with retroviruses that direct the expression of Bcl-2 and LacZ. These marked (LacZ-positive), Bcl-2-expressing cells were mixed in varying proportions with unmarked control cells, and the mixtures were subjected to multiple cycles of killing by exposure to proapoptotic conditions followed by rescue and expansion of resistant cells under normal growth

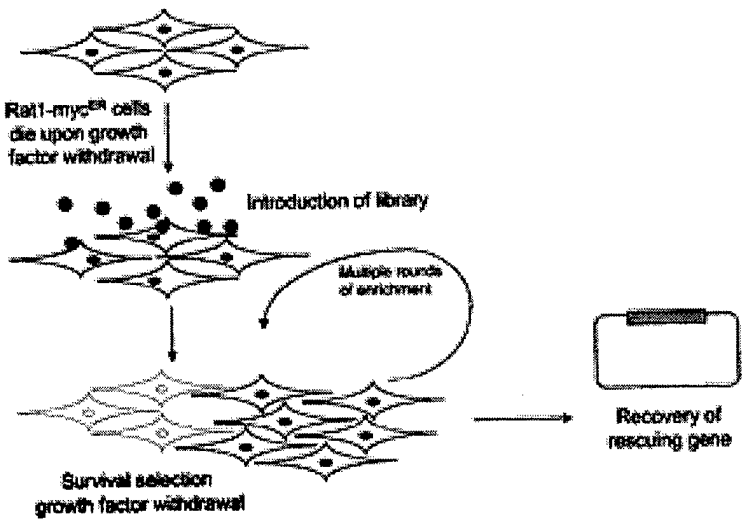

Figure 1. Schematic outline of the screen designed to identify genes that confer protection from Myc-induced apoptosis.

conditions. The proportion of LacZ/Bcl-2 cells was monitored following each round, and after four cycles, β-galactosidase staining indicated that Bcl-2-expressing cells had been enriched ~50,000-fold from a frequency of $1/10^5$ to a frequency of 1/2. Because we reasoned that other antiapoptotic genes might not be as effective as *bcl-2*, we designed the selection procedure to allow recovery of genes that were enriched a minimum of 1,000-fold by four killing cycles.

Populations of Rat1/MycER cells were infected with a retroviral cDNA library that had been prepared from cells that were committed to apoptosis. Individual plates containing ~5×10^6 cells were infected with pools containing from 10,000 to 100,000 distinct cDNAs. This insured that each individual cDNA was represented multiple times in the starting cell population. Infected cell pools were subjected to four cycles of enrichment by the apoptosis/rescue protocol that we had established using Bcl-2 as a model. Proviruses were recovered from populations that had been enriched for resistant cells, and highly represented genes were identified by fingerprinting 50 cDNAs from each population. Most of the pools contained cDNA clones that had been clearly enriched by the procedure.

The genetic strategy was validated by the isolation of cDNAs encoding proteins that had been shown previously to protect from apoptosis. For example, we isolated many (9) independent cDNAs encoding Mcl-1 a member of the Bcl-2 family that prevents cell death and promotes differentiation in hematopoietic cells (Kozopas et al. 1993). Furthermore, we identified as a protective protein glutathione peroxidase, an enzyme that can counteract apoptosis induced by reactive oxygen species (Hockenbery et al. 1993). We also obtained two independent clones of Ha-*ras*. Expression of *ras* has been shown recently to prevent apoptosis in Rat1/MycER cells via activation of the AKT pathway (Kauffmann-Zeh et al. 1997). All three of these genes protected Rat1/MycER cells from apoptosis under our experimental conditions (not shown).

Twist and Dermo1 prevent Myc-dependent cell death

In addition to known antiapoptotic genes, we also isolated a large number of potentially protective cDNAs that had not been shown previously to antagonize cell death. Among these were multiple independent isolates encoding two closely related proteins, Twist and Dermo1. *twist* was represented by two independent cDNAs whereas *dermo1* was isolated four times.

Twist was originally identified in *Drosophila* as a protein involved in establishing dorso–ventral polarity (Thisse et al. 1987). Dermo1 emerged from a two-hybrid screen for tissue-specific factors that could interact with the ubiquitous bHLH protein, E12. Twist and Dermo1 belong to the basic–helix–loop–helix (bHLH) family of transcription factors and are quite similar (>90% identity) in the bHLH and carboxy-terminal domains. The amino termini are less closely related; Dermo1 lacks a glycine-rich region that is present in Twist (Li et al. 1995). Although specific transcriptional targets of Twist and Dermo1 have not yet been identified in mammals, expression patterns in *Drosophila*, *Xenopus*, and mouse suggest an involvement in the regulation of diverse developmental processes, particularly in the formation of mesoderm (Futchbauer et al. 1995; Li et al. 1995; Gitelman 1997).

The enrichment of two such closely related proteins during the genetic selection and the isolation of each from multiple, independent cell pools prompted a deeper investigation of the possibility that Twist and Dermo1 could interfere with oncogene-induced apoptosis. Expression of either Twist or Dermo1 reduced the number of apoptotic cells that were observed following either Myc activation or serum withdrawal to ~50% of the number observed in control (LacZ-infected) or uninfected populations (Fig. 2). Similar levels of protection were evident from an analysis of viable cells that remain following serum depletion (Fig. 2). As a complement to these assays, we quantified a biochemical marker of apoptosis following Myc activation. Twist expression reduced levels of active CCP32 (caspase-3) to about one-half of those detected in control, LacZ-expressing cells (data not shown). In aggregate, these results demonstrate that both Twist and Dermo1 can protect from oncogene-induced apoptosis.

In addition to its ability to protect from acute apoptotic stimuli, Twist also conferred long-term protection. Both Rat1 and Rat1/MycER cells die on prolonged (~3 weeks) serum starvation. Expression of Twist not only delayed the appearance of apoptotic cells but also prevented cell death in a significant percentage of infected cells (Fig. 2; data not shown). In longer term assays, Twist was indistinguishable from Bcl-2 in the ability to prevent cell death. Dermo1 was similar to Twist in its ability to protect from both acute and long-term proapoptotic stimuli.

Twist and Dermo1 could potentially protect from programmed cell death through a variety of mechanisms. Because Twist and Dermo1 share features of transcription factors, we asked whether ectopic expression of these proteins affected the abundance of known antiapoptotic proteins. We found no evidence for changes in the levels of several members of the *bcl-2* family, nor did we observe altered expression of the MycER protein that provided the proapoptotic stimulus in these cells. Twist and other bHLH transcription factors have been implicated in the control of diverse developmental processes. We therefore tested the possibility that ectopic expression of Twist or Dermo1 might alter the identity of fibroblasts in a manner that increased resistance to *myc*-induced cell death. However, Twist-infected fibroblasts maintained their original morphology and retained the expression of a constellation of markers that is characteristic of the fibroblastic lineage (data not shown).

Twist antagonizes p53

The tumor suppressor *p53* plays a critical role in regulating cell death in response to a variety of stimuli. In

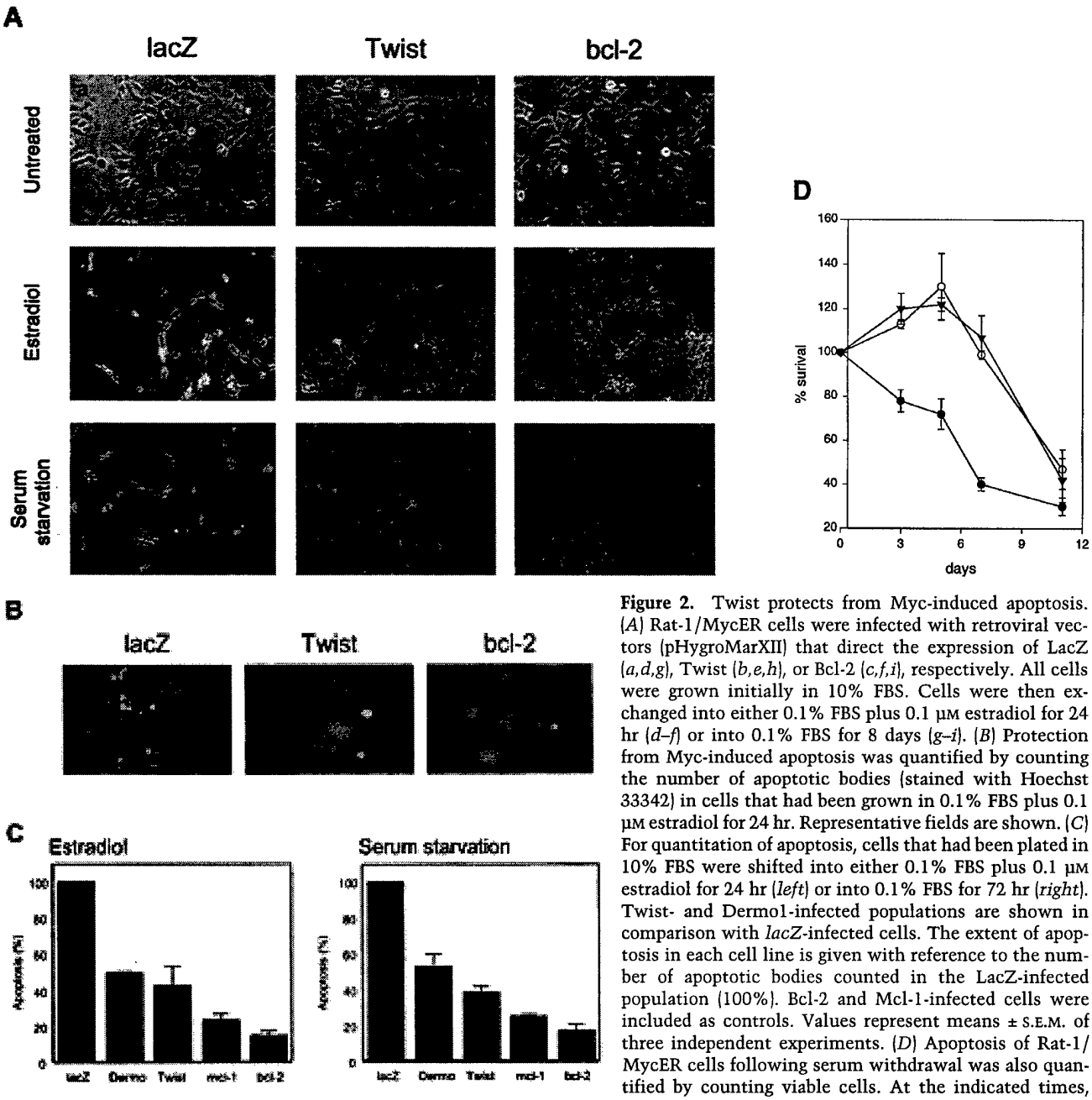

Figure 2. Twist protects from Myc-induced apoptosis. (*A*) Rat-1/MycER cells were infected with retroviral vectors (pHygroMarXII) that direct the expression of LacZ (*a,d,g*), Twist (*b,e,h*), or Bcl-2 (*c,f,i*), respectively. All cells were grown initially in 10% FBS. Cells were then exchanged into either 0.1% FBS plus 0.1 μM estradiol for 24 hr (*d–f*) or into 0.1% FBS for 8 days (*g–i*). (*B*) Protection from Myc-induced apoptosis was quantified by counting the number of apoptotic bodies (stained with Hoechst 33342) in cells that had been grown in 0.1% FBS plus 0.1 μM estradiol for 24 hr. Representative fields are shown. (*C*) For quantitation of apoptosis, cells that had been plated in 10% FBS were shifted into either 0.1% FBS plus 0.1 μM estradiol for 24 hr (*left*) or into 0.1% FBS for 72 hr (*right*). Twist- and Dermo1-infected populations are shown in comparison with *lacZ*-infected cells. The extent of apoptosis in each cell line is given with reference to the number of apoptotic bodies counted in the LacZ-infected population (100%). Bcl-2 and Mcl-1-infected cells were included as controls. Values represent means ± S.E.M. of three independent experiments. (*D*) Apoptosis of Rat-1/MycER cells following serum withdrawal was also quantified by counting viable cells. At the indicated times, adherent and nonadherent populations were collected and stained with trypan blue. Numbers of viable cells are given with reference to the starting populations. (●) Gal; (○) Twist; (▼) Dermo1. Values represent means ± S.D. of three independent experiments.

fact, Myc-induced cell death has a clearly demonstrated dependence on p53 in a number of experimental systems (Hermeking and Eick 1994; Wagner et al. 1994). Thus, we examined the possibility that Twist might protect from apoptosis by damping the p53 response. Although Rat1/MycER cells express wild-type p53, the proapoptotic role of p53 in these cells is not well established. We therefore used a cell line in which apoptosis has a demonstrated dependence on p53 function.

MEFs that express both E1A and Ha-RasV12 (C8 MEF; Lowe et al. 1993,1994) execute a cell death program in response to a variety of insults; among these are DNA damage, growth factor deprivation, and contact inhibition. This apoptosis is strictly p53-dependent, because analogously engineered MEFs derived from *p53*-null mice do not die under identical conditions (Lowe et al. 1993). As was also observed for Bcl-2, ectopic expression of Twist dramatically delayed apoptosis following adriamycin treatment of C8 MEFs (Fig. 3B). Moreover, Twist–C8 MEF resisted serum starvation and contact inhibition; a significant population of Twist–C8 MEF survived in the long term (~2 weeks), whereas essentially none of

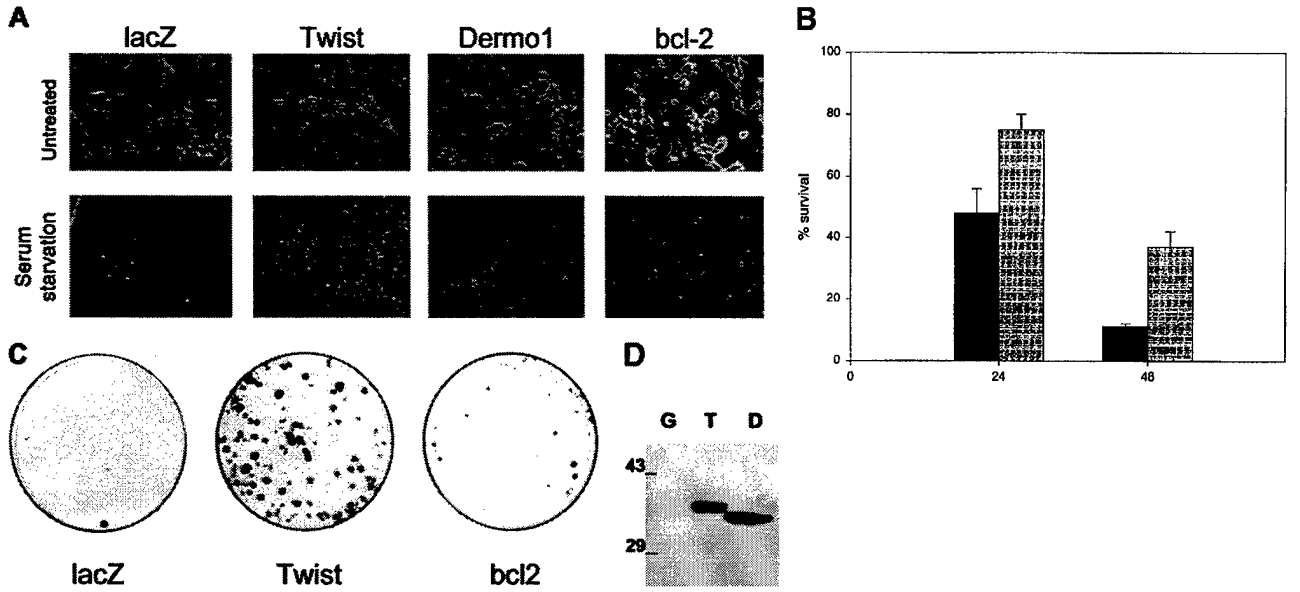

Figure 3. Twist antagonizes p53. (*A*) E1A-Ras expressing MEFs (C8 MEFs) were infected with a retroviral vector (pBabe-puro) encoding LacZ, Twist, Dermo1, or Bcl-2, as indicated. Cells were plated in the presence of 10% FBS and after 24 hr were shifted to 0.1% FBS for 5 days. The result of a representative experiment is shown. Similar results were obtained after adriamycin-treatment (0.2 μg/ml) and after contact inhibition. (*B*) C8 MEFs infected with retroviruses that direct the expression of either Twist (shaded bars) or β-gal (solid bars) were treated with 0.2 μg/ml adriamycin for the indicated times. Viable cell numbers were determined by trypan blue staining and normalized to the starting cell number for each culture (100%). Values represent means ± S.D. for four independent experiments from two independent infections. (*C*) MEF-A3s were infected with retroviral vectors (pBabe-puro) that direct the expression of LacZ, Twist, or Bcl-2. After drug selection, cells were plated at low density and shifted to the permissive temperature of 32°C. Colony formation was monitored after 10 days. (*D*) C8 MEFs were infected with a control, LacZ (G) virus, or with viruses that direct the expression of Myc-tagged Twist (T) or Dermo1 (D). Protein expression was verified by Western blotting with a monoclonal antibody to the Myc tag (9E10).

the LacZ-infected control cells survived (Fig. 3A). Similar levels of protection were afforded by ectopic expression of Dermo1 (Fig. 3).

Considered as a whole, our data suggested that the antiapoptotic effects of Twist and Dermo1 may result, at least in part, from antagonism of the p53 pathway. We therefore asked whether Twist could interfere with other aspects of p53 function. Specifically, we probed the effect of Twist on p53-mediated growth arrest. Embryo fibroblasts from a p53-null mouse were engineered to express a temperature-sensitive version of p53 (p53Val135; MEF-A3). MEF-A3 can be continuously grown at 39°C, a temperature at which p53 assumes a nonfunctional conformation. However, at the permissive temperature of 32°C, the ectopically expressed p53 assumes a wild-type conformation, and cells reversibly arrest in the G_1 phase of the cell cycle (Michalovitz et al. 1990). MEF-A3s were infected either with a retrovirus that directs Twist expression or with a control (LacZ) virus. Upon shift to 32°C, control cells arrested and failed to form colonies, whereas a significant percentage of Twist-infected cells continued to proliferate and formed colonies (Fig. 3C). The penetrance of this bypass was similar to that seen on expression of a highly effective p53 antisense RNA (A. Carnero, D. Beach, and G. Hannon, unpubl.). In contrast, MEF-A3 cells infected with a Bcl-2 virus did not form colonies (Fig. 3C). This suggests a specific antagonism of p53 by Twist rather than rescue of colony formation as a secondary consequence of the ability of Twist to protect from apoptosis.

The ability of p53 to transactivate target genes is key for efficient induction of growth arrest and apoptosis (Attardi et al. 1996; Chen et al. 1996). Therefore, we tested whether Twist interfered with the ability of p53 to function as a transcriptional activator. Increasing amounts of a Twist expression construct were transfected into p53-null MEFs in combination with fixed amounts of a p53 expression vector and a p53-dependent reporter. In a dose-dependent manner, Twist suppressed transcription from a synthetic, p53-responsive promoter (PG-13) but had no effect on nonresponsive promoters (Fig. 4A; data not shown). Similar effects were observed in U2OS cells wherein the synthetic p53-responsive promoter depends on endogenous p53 for its activity (Fig. 4B).

Because Twist could interfere with the transcription of a p53-dependent reporter, we probed the effects of ectopic Twist expression on the induction of p53 target genes. For these experiments, we used C8 cells in which Twist had been shown to antagonize p53-dependent apoptosis. Control cells, infected with a β-galactosidase retrovirus, induce *p21*, *bax*, and *MDM2* mRNAs on treatment with adriamycin, a DNA damaging agent that provokes a p53 response (Fig. 4C, lanes G). In contrast, Twist-expressing cells fail to induce *p21* and induce *MDM2* to a lesser extent than do control cells (Fig. 4C, lanes T). Induction of *bax* is also impaired in Twist ex-

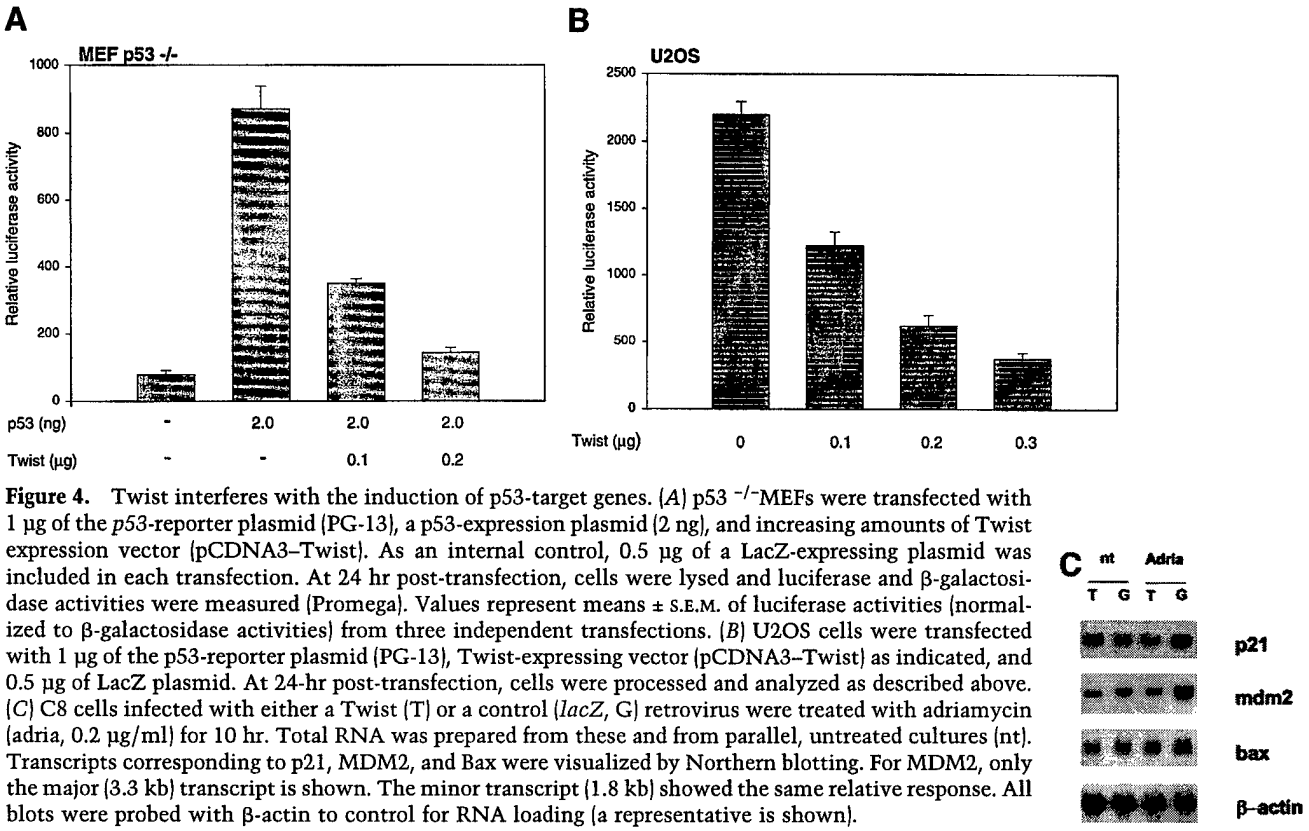

Figure 4. Twist interferes with the induction of p53-target genes. (*A*) p53 $^{-/-}$MEFs were transfected with 1 μg of the *p53*-reporter plasmid (PG-13), a p53-expression plasmid (2 ng), and increasing amounts of Twist expression vector (pCDNA3–Twist). As an internal control, 0.5 μg of a LacZ-expressing plasmid was included in each transfection. At 24 hr post-transfection, cells were lysed and luciferase and β-galactosidase activities were measured (Promega). Values represent means ± S.E.M. of luciferase activities (normalized to β-galactosidase activities) from three independent transfections. (*B*) U2OS cells were transfected with 1 μg of the p53-reporter plasmid (PG-13), Twist-expressing vector (pCDNA3–Twist) as indicated, and 0.5 μg of LacZ plasmid. At 24-hr post-transfection, cells were processed and analyzed as described above. (*C*) C8 cells infected with either a Twist (T) or a control (*lacZ*, G) retrovirus were treated with adriamycin (adria, 0.2 μg/ml) for 10 hr. Total RNA was prepared from these and from parallel, untreated cultures (nt). Transcripts corresponding to p21, MDM2, and Bax were visualized by Northern blotting. For MDM2, only the major (3.3 kb) transcript is shown. The minor transcript (1.8 kb) showed the same relative response. All blots were probed with β-actin to control for RNA loading (a representative is shown).

pressing cells; however, effects on this gene are more subtle than are those observed for other targets. In these same cells, neither the basal level of p53 protein or mRNA nor the extent to which p53 protein was induced following adriamycin treatment was influenced by ectopic Twist expression (data not shown).

A potential mechanism underlying regulation of p53 by Twist

Recent evidence suggests that oncogenes such as *myc* and *E1A* sensitize cells to p53-dependent cell death, at least in part, through effect on the ARF tumor suppressor (de Stanchina et al. 1998; Zindy et al. 1998). ARF is an upstream regulator of p53 that acts through effects on the localization and activity of MDM2 (Honda and Yasuda et al. 1998; Zhang et al. 1998; Weber et al. 1999; Zhang and Xiong 1999). Expression of either E1A or Myc in primary MEFs provoked substantial increases in *ARF* mRNA levels (de Stanchina et al. 1998; Zindy et al. 1998), leading, in turn, to activation of the p53 pathway and to consequent induction of downstream targets such as *p21* and *MDM2*. The p53 pathway failed to respond to E1A or Myc in *ARF*-null cells, placing ARF as a key mediator of homeostatic responses to oncogene expression. Therefore, we asked whether Twist expression had any effect on ARF.

C8 cells that have been engineered to ectopically express Twist show a dramatic reduction in *ARF* mRNA as compared with control (LacZ-expressing) cells (Fig. 5). This down-regulation is striking considering that loss of p53 function such as is observed in the Twist-expressing cells normally results in substantial increases in the abundance of the *ARF* transcript (Quelle et al. 1995).

Down-regulation of *ARF* provides a potential mechanism by which Twist may affect p53 function. *ARF*-null MEFs are resistant to p53-induced growth arrest, and ectopic expression of p53 in these cells does not activate

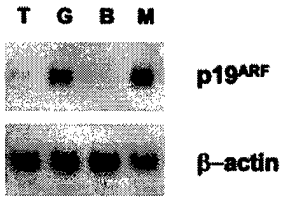

Figure 5. Twist down-regulates *ARF*. RNA was prepared from C8 cells infected with retroviruses that direct the expression of either Twist (T) or LacZ (G). *ARF* transcript was visualized by Northern blotting with an *ARF*-specific probe (exon 1β). For comparison, RNA was also prepared from BALBc 3T3 cells (B) that lack *ARF* expression and from primary MEFs (M) that are known to express *ARF* at high levels. The same blot was also probed for mouse β-actin as a control. Identical results are obtained from multiple independent infections and with cells plated under a variety of different conditions. Induction of apoptosis by treatment with adriamycin had no effect on the reduction of *ARF* mRNA by Twist.

the *p21* promoter (Kamijo et al. 1998). Furthermore, MEFs lacking *ARF* exhibit a reduced apoptotic response to *myc* and *E1A*. Disruption of ARF led to an ~50% reduction in cell death in *myc*-expressing MEFs that had been deprived of serum survival factors (Zindy et al. 1998). Resistance was not as complete as was achieved on disruption of *p53* itself, indicating that ARF-independent mechanisms also contribute to factor-dependant survival. Similarly, *ARF* disruption in E1A-expressing MEFs led to a decreased sensitivity to adriamycin (de Stanchina et al. 1998). In both cases, the response of p53 targets was attenuated, although not all were affected to the same degree. Thus, cells that express Twist ectopically share many features with *ARF*-null MEFs. However, in Twist-expressing cells, some *ARF* mRNA persists. Furthermore, Twist is undoubtedly a multifunctional protein. Therefore, the consequences of *ARF* loss and ectopic Twist expression are unlikely to perfectly overlap.

Relatively little is known about the regulation of ARF expression. It is still unclear whether induction of *ARF* by oncogenes such as *myc* and *E1A* is direct or results from secondary effects on transcriptional regulators such as E2F-1 that also modulate *ARF* transcription (DeGregori et al. 1997; Bates et al. 1998; Robertson and Jones 1998). Similarly, we do not yet know whether down-regulation of *ARF* by Twist is mediated through an effect on the *ARF* promoter or through an indirect route.

Although decreases in ARF may be sufficient to explain the observed effects of Twist on p53, we cannot exclude that additional mechanisms may also contribute. The activity of p53 is tightly controlled by a complex series of pathways that are interconnected by feedback loops. Both the synthesis and degradation of the p53 protein are regulated in response to inducing stimuli (for review, see Ko and Prives 1996; Agarwal et al. 1998). In addition, post-translational modifications such as acetylation and phosphorylation as well as an association with cofactors regulate the stability and the specific activity of this transcription factor (Haupt et al. 1997; Kubbutat et al. 1997; Shieh et al. 1997; Siciliano et al. 1997). In particular, the activity of p53 can be modulated by its interaction with the coactivator p300/CBP. p300/CBP is an acetyltransferase that can modify p53 and alter its ability to bind target sequences in vitro (Avantaggiati et al. 1997; Gu et al. 1997; Lill et al. 1997). Moreover, through its interaction with MDM2, p300 has been linked to p53 degradation (Grossman et al. 1998). Twist has been shown recently to interact physically with p300 and to inhibit acetyltransferase activity in an in vitro assay (Hamamori et al. 1999). Thus, we cannot rule out the possibility that Twist may also modulate p53 activity through effects on p300/CBP and related partners.

twist *is a candidate oncogene product for rhabdomyosarcoma*

Increased resistance to programmed cell death, disruption of the INK4/*ARF* locus, and loss of p53 activity are common characteristics of human tumor cells. Expression of Twist can prevent apoptosis, down-regulate ARF, and interfere with p53 function. We therefore examined the possibility that *twist* might have properties that are characteristic of oncogene products.

Loss of anchorage dependence is a hallmark of tumor cells, and the ability to promote anchorage-independent growth is a common property of oncogenes. Some transformed cells, such as fibroblasts that express either a combination of Ras and Myc or a combination of Ras and E1A (e.g., C8 MEFs), show a low efficiency of anchorage-independent growth. This failure is probably due to a predisposition to apoptosis because inhibition of programmed cell death through loss of *p53*, inactivation of the apoptotic machinery, or expression of protective oncoproteins such as Bcl-2 can promote colony formation (Nikiforov et al. 1996; Soengas et al. 1999). Therefore, we tested whether expression of either Twist or Dermo1 could allow the growth of C8 cells in soft agar. In accord with previous reports, C8 cells infected with a control, LacZ retrovirus form a few small colonies in semisolid media (Fig. 6). In contrast, expression of either Twist or Dermo1 stimulates formation of robust colonies in soft agar. Similar results are obtained on expression of either Bcl-2 (Fig. 6) or a dominant, interfering allele of p53 (Nikiforov et al. 1996). These results indicate that *twist* and *dermo1* share one property of oncogene products, the ability to promote anchorage-independent growth.

To assess the possibility that aberrant *twist* expression might be a feature of human cancers, a variety of human tissue and tumor samples (archivally preserved primary patient material) were tested for the abundance of Twist protein. Consistent with studies on mouse embryos (Futchbauer 1995; Gitelman 1997), antibodies raised

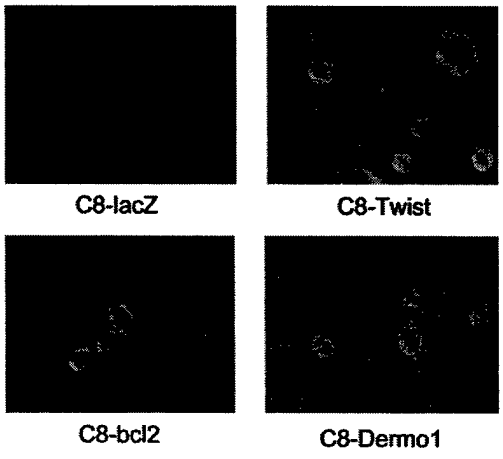

Figure 6. Twist and Dermo1 promote colony formation in soft agar. C8 MEFs were infected with retroviruses that direct the expression of LacZ, Twist, Dermo1, or Bcl-2. Infected cells were plated in soft agar, and colony formation was assessed after 2 weeks (as indicated). The LacZ-expressing cells form only a few, small colonies. Cells infected with Twist, Dermo1, or Bcl-2 form large colonies similar to those that are observed on expression of dominant-interfering alleles of p53. Expression of Twist or Dermo1 enhances colony formation by approximately five to sevenfold.

against either the amino or the carboxyl terminus of Twist gave a specific nuclear staining pattern in human embryonic mesenchymal tissues (not shown). Twist protein was not detectable in a panel of common epithelial tumors such as those of the breast, colon, ovary, and lung. However, high-level Twist expression was seen in ~50% of rhabdomyosarcomas (8/15 cases analyzed). In Twist-positive tumors, antisera directed against either the amino or carboxyl terminus decorated a percentage of neoplastic cells ranging between 60% and 90%. Surrounding normal tissues and Twist-negative rhabdomyosarcomas displayed no detectable Twist immunoreactivity (Fig. 7).

Rhabdomyosarcomas constitute a heterogeneous group of malignant tumors, mainly affecting children, that originate from undifferentiated mesenchymal cells. In rhabdomyosarcoma cells, skeletal muscle differentiation is arrested at an early stage despite the expression of myogenic markers, such as myoD and myogenin (Pappo 1996). It has been well established that Twist is excluded from the developing myotome and is not expressed in differentiated skeletal muscle (Fig. 7d; Futchbauer 1995; Gitelman 1997). These observations led to in vitro experiments that demonstrated the ability of Twist to block myogenic differentiation in cultured cells (Spicer et al. 1996; Hamamori et al. 1997; Hebrok et al. 1997). Therefore, our finding of inappropriate Twist expression in rhabdomyosarcomas suggests that Twist may have multiple roles in the formation of these tumors. First, Twist expression might halt the developmental program that leads to terminal differentiation and withdrawal of muscle cell precursors from the division cycle. Second, Twist expression might antagonize apoptosis. Third, Twist may interfere with the p53 tumor suppressor pathway, the loss of which is one of the most common genetic alterations in human tumors.

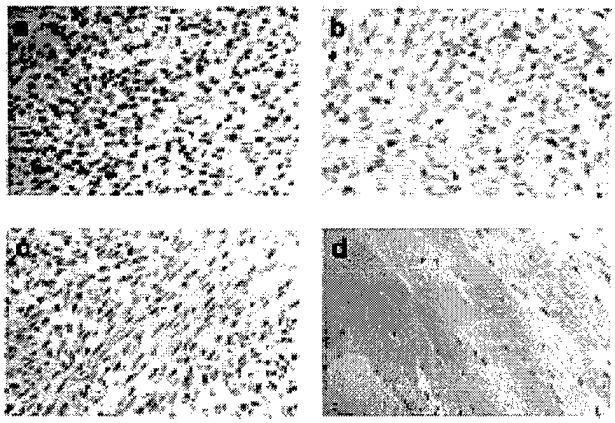

Figure 7. Twist is highly expressed in rhabdomyosarcomas. (*a*) Formalin-fixed histologic sections of a rhabdomyosarcoma were stained with a Twist-specific antibody (SC-6070). Most of the neoplastic cells show nuclear accumulation of Twist protein. (*b*) Specificity of the signal was confirmed by loss of Twist immunoreactivity after preincubation of Twist antibody with the antigenic peptide. (*c*) A representative stain of a Twist-negative rhabdomyosarcoma is shown. (*d*) The lack of Twist immunoreactivity in differentiated skeletal muscle. Original magnification 400×.

Although inappropriate expression of Twist may have a role in the genesis of some tumors, reduction of Twist activity can also have dramatic consequences for a developing organism. In *Drosophila*, *Xenopus*, and mouse, *twist* is essential for mesoderm formation (Chen and Behringer 1995; Futchbauer 1995; Thisse et al. 1995; Gitelman 1997). *twist*-null mice die at day 11.5 postcoitum. Just prior to death, these animals show a massive wave of apoptosis in the developing somites, a site in which Twist is normally expressed (Chen and Behringer 1995). Alterations in Twist activity have also been linked to developmental abnormalities in humans. Mutations in the *twist* gene have been causatively linked to Saethre–Chotzen syndrome (el Gouzzi et al. 1997; Howard et al. 1997), a hereditary disorder characterized by a variety of limb and craniofacial anomalies. Of these, craniosynostosis is the most striking. This malformation of the skull is caused by premature fusion of cranial sutures. It has long been proposed that many craniosynostosis syndromes result from local perturbation of apoptotic programs that are essential for proper timing of suture fusion (Bourez et al. 1997). Considered together, the phenotype of organisms with altered Twist activity is consistent with a role for Twist in regulating apoptosis during development. Rhabdomyosarcoma cells may exploit this normal function of Twist to counteract the proapoptotic stimuli that result from oncogene activation.

Materials and methods

Cells

Rat-1/MycER cells that express an estrogen-inducible *myc* gene (Evan et al. 1992) were maintained at 5% CO_2 in DMEM without phenol red (wDMEM), supplemented with 10% FBS. C8 MEF cells (mouse embryo fibroblasts that express E1A and H-RasVal12) (Lowe et al. 1993) and the ecotropic packaging cell line, LinX (Hannon et al. 1999), were maintained in 5% CO_2 in DMEM, supplemented with 0.01% Na pyruvate and 10% FBS. MEF-A3 cells were produced by infecting MEFs derived from *p21/p53*-null mice with a retroviral vector that directs the expression of a temperature-sensitive mutant of p53, $p53val^{135}$. A resulting clonal cell line (MEF-A3) that rapidly arrested after p53 induction at the permissive temperature (32°C) was used for colony formation analysis. MEF-A3s were grown in 5% CO_2 in DMEM supplemented with 0.01% Na pyruvate and 10% FBS at 39°C.

Library construction and screen strategy

Poly(A)$^+$ RNA was extracted by the use of Triazol reagent (GIBCO–BRL), from Rat-1/MycER cells committed to apoptosis by 6 hr of serum starvation. Oligo(dT)-primed cDNA was produced with the Stratagene ZapII cDNA synthesis kit (Hannon et al. 1993). Fragments were cloned into the retroviral expression vector pHygroMaRXII at the *Eco*R1 and *Xho*I sites (Hannon et al. 1999). The library was divided into 100 independent DNA plasmid pools, each with a complexity of 10^4–10^5 clones. Each plasmid pool was used to transfect LinX E packaging cells by the

calcium phosphate method. At 72-hr post-transfection, viral supernatants were collected, filtered, supplemented with 4 μg/ml Polybrene, and used to infect Rat-1/MycER cells. After infection, cells were selected with hygromicin and then subjected to an apoptosis/rescue schedule as described: A total of 5×10^5 Rat-1/MycER cells were plated onto 10-cm dishes. At 24-hr postplating, apoptosis was induced by adding fresh medium supplemented with 0.1% FBS plus 0.1 μM estradiol for 3 days, followed by 2 days of culture in serum starvation without estradiol. Cells were then refed with medium containing 10% FBS. Rescued cells were then replated at low density and subjected to another cycle of killing as above. Cycles of killing/rescue were repeated four times.

Recovery of viral genomes and analysis of the recovered plasmids

Genomic DNA was extracted from cell populations that had been enriched for resistance to apoptosis by a standard proteinase K/SDS method. Five micrograms of genomic DNA were treated with CRE recombinase, phenol extracted, ethanol precipitated, and used to transform electrocompetent bacteria. Fifty recovered plasmids were analyzed from each pool by restriction digestion. Those plasmids that represented >5% of the recovered plasmid species were sequenced. A subset of these was again introduced into Rat1/MycER cells and tested for the ability to protect from apoptosis.

Quantitation of apoptosis in Rat-1/MycER cells

Rat-1/MycER cells infected with pHygroMarX retroviral vectors that direct the expression of the Twist, Dermo1, Bcl-2, and LacZ, respectively, were plated at low density (10^5 cells/well) onto acid-treated coverslips in 6 well plates. Twenty-four hours after plating, cells were washed twice with PBS and then induced to apoptose. Myc-induced apoptosis was triggered by treatment with 0.1 μM estradiol in 0.1% FBS. Apoptosis was monitored 24 hr post-induction. Growth factor deprivation-induced apoptosis was triggered by replacing the culture medium with fresh DMEM supplemented with 0.1% FBS. The extent of apoptosis was quantified at 72 hr post-treatment by Hoechst staining (Attardi et al. 1996). Briefly, cells were directly stained with 4 μ/ml of Hoechst 33342 for 10 min, washed with PBS, and mounted. At least 100 fields/slide were analyzed and the number of apoptotic bodies was evaluated blind by two independent observers.

Moreover, protection from apoptosis was also analyzed by trypan blue exclusion. Cells were seeded into six-well plates (10^5/ well) 24 hr prior to serum withdrawal. At various times, adherent and nonadherent cells were pooled and viability assessed by trypan blue exclusion.

Apoptosis in C8 MEFs

MEFs that express E1A and H-RasVal12 (C8 MEFs) were infected with retroviral vectors (pBABE-Puro) that drive the expression of LacZ, Bcl-2, Twist, Dermo1, or Myc-tagged versions of Twist and Dermo1. In all assays, Myc-tagged Twist and Dermo1 were indistinguishable from the untagged proteins. Twist and Dermo1 protein expression in C8 MEFs was confirmed by Western blotting with a monoclonal antibody to the Myc tag (9E10).

After selection, cells were plated at low density and maintained in complete media to monitor the cell–cell contact-triggered apoptosis, subjected to serum starvation (0.1% FBS) for 5 days, or treated with adriamycin (0.2 μg/ml) for 2 days.

Cell viability after adriamycin treatment was assessed by trypan blue exclusion. Briefly, cells were seeded into six-well plates (10^5/ well) 48 hr prior to drug treatment (adriamycin, 0.2 μg/ml). At various times, adherent and nonadherent cells were pooled and a trypan blue exclusion test performed.

RNA extraction and Northern blot analysis

Total RNA was extracted from C8 MEFs infected with pBABE-*lacZ* and pBABE-Twist expression vectors in normal growth conditions and after induction of apoptosis by adriamycin treatment (0.1 μg/ml) for 10 hr. Triazol reagent (GIBCO-BRL) was used according to the manufacturer's instructions. An additional final precipitation in LiCl was performed to further purify RNA from contaminant DNA. Briefly, after Triazol extraction the RNA pellet was resuspended in 5 vol of 100 mM HEPES (pH 7.5) and the same volume of 5 M LiCl was added drop-wise. Precipitation was performed at −20°C for at least 4 hr.

For Northern blots, 10 μg of total RNA was loaded per lane and fractionated in a 1% agarose/formaldehyde gel. After transfer onto Hybond N+ membrane (Amersham), blots were hybridized with ^{32}P-labeled probes specific for mouse *p21*, *mdm2*, *p19^{ARF}* (exon Iβ), and human *bax* genes. A probe specific for mouse *β-actin* was used to confirm equal loading. Membranes were hybridized overnight at 65°C in 0.2 M $NaPO_4$, 1 mM EDTA, 7% SDS, and 1% BSA in the presence (mouse probes) or absence (human *bax* probe) of 15% formamide. Membranes were washed twice in 0.1% SDS, 0.2× SSC and once in 0.1× SSC at 60°C, followed by autoradiography.

Bypass of p53-induced growth arrest

MEF-A3 cells that express a temperature-sensitive version of p53 (p53val^{135}) were infected with pBABE-Puro vectors that drive the expression of LacZ, Twist, or Bcl-2, respectively. After selection, 5×10^4 cells were plated in quadruplicate in 10 cm plates. On the following day, two plates were shifted to the permissive temperature (32°C). The remaining two plates were used as controls for plating efficiency. After 10–15 days, colony formation was scored by crystal violet staining.

Cell transfections and transcription assays

Transfections of MEF $p53^{-/-}$ and U2OS cells were performed according to the calcium phosphate precipitation protocol as described (Hamamori et al. 1997). A total of 9 μg of plasmid DNA per 6-cm-diam. dish was used. As an internal control, 0.5 μg of a LacZ-expressing plasmid was included in each transfection. At 20 hr post-transfection, cells were refed, incubated for 2 additional days, and harvested for reporter gene assays. Luciferase and β-galactosidase activities were measured by a MLX microtiter plate luminometer (Dynex, Chantilly, VA). Values represent means ± S.E.M. of luciferase activities (normalized to β-galactosidase) from at least three independent transfections performed in duplicate.

Anchorage-independent growth in C8 MEFs

C8 MEFs infected with retroviral vectors (pBABE-Puro) that drive the expression of LacZ, Twist, Dermo1, or Bcl-2, were analyzed for anchorage-independent growth in semi-solid medium. Approximately 10^5 cells were plated in 0.3% low-melting-point agarose/growth medium onto 60-mm dishes with a 0.5% agarose underlay. Colonies were photographed after 2 weeks.

Immunohistochemistry

A series of common human tumors including 10 gastric and colorectal carcinomas, 6 breast, 10 lung, and 4 ovarian carcinomas, 2 Kaposi's sarcomas, 3 melanomas, 8 leiomyosarcomas, and 15 rhabdomyosarcomas were analyzed by immunohistochemistry for Twist expression with an avidin–biotin–peroxidase complex (ABC) technique. Formalin-fixed histologic sections were incubated with an affinity-purified goat polyclonal antibody raised against a peptide corresponding to an amino acid sequence at the amino terminus of human Twist (SC-6070, Santa Cruz Biotechnology; dilution, 0.2 μg/ml) or with an affinity-purified goat polyclonal antibody raised against a peptide corresponding to an amino acid sequence mapping at the carboxyl terminus of human Twist (SC-6269, Santa Cruz Biotechnology, dilution: 0.2 μg/ml). The primary antibodies were incubated at +4°C overnight. Immunoreaction was visualized with a biotin-conjugated anti-goat antiserum followed by peroxidase–streptavidin and DAB chromogen development. The specificity of the SC-6070 antiserum was also confirmed by an adsorption test. Peptide sc 6070p (SantaCruz) corresponding to the amino acid sequence of the amino terminus of human Twist (1 μg/ml) was incubated with the anti-Twist goat polyclonal antibody (0.2 μg/ml) for 2 hr at room temperature before immunostaining. The percentage of immunoreactive cells was evaluated by scanning sections and counting at least 1000 neoplastic cells.

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Appendix 4

A Proinflammatory Cytokine Inhibits p53 Tumor Suppressor Activity

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Summary

p53 has a key role in the negative regulation of cell proliferation, in the maintenance of genomic stability, and in the suppression of transformation and tumorigenesis. To identify novel regulators of p53, we undertook two functional screens to isolate genes which bypassed either p53-mediated growth arrest or apoptosis. In both screens, we isolated cDNAs encoding macrophage migration inhibitory factor (MIF), a cytokine that was shown previously to exert both local and systemic proinflammatory activities. Treatment with MIF overcame p53 activity in three different biological assays, and suppressed its activity as a transcriptional activator. The observation that a proinflammatory cytokine, MIF, is capable of functionally inactivating a tumor suppressor, p53, may provide a link between inflammation and tumorigenesis.

Key words: macrophage migration inhibitory factor • p53 • inflammation and cancer • growth arrest • apoptosis • senescence

Elucidating the molecular mechanisms of tumorigenesis is essential for future progress in the diagnosis and treatment of human cancer. Inactivation of tumor suppressor genes is an essential step in the etiology of tumor initiation and growth. A great deal of effort has focused on the role of the p53 tumor suppressor in cancer (1, 2). Its pivotal position is underscored by the observation that mutations in p53 are the most common genetic alteration in human tumors.

p53 has a key role in inducing growth arrest or apoptosis after genotoxic stress (3–8). Cells lacking p53 are capable of proliferation with damaged DNA, and thus are capable of accumulating multiple, potentially oncogenic mutations (9, 10). In addition, p53 controls the onset of cellular senescence, a process which limits the number of times a cell can potentially divide and which may act as an antitumor mechanism (11). Overcoming p53 function extends potential life span and directly contributes to cellular immortalization (12–14).

In a variety of tumors, p53 is functionally inactivated, but the gene remains intact (15–17). In these tumors, the activity of p53 regulators may be altered. Thus, the identification and characterization of novel regulators of p53 activity may have direct consequences for understanding the etiology of multiple tumor types.

Eventual tumor formation has been associated with several chronic inflammatory conditions, although the relationship between inflammation and tumor development remains largely obscure at a molecular level (18, 19). Tumor initiation is precipitated by a combination of oncogenic mutational events and loss of the cellular controls that prevent cell division in the presence of DNA damage, leading to fixation and propagation of these mutations (9). At sites of inflammation, the release of reactive oxygen species from activated phagocytes has been associated with genotoxic damage in adjacent cells (20, 21). However, it has been unclear how these cells could bypass the normal controls to prevent proliferation with damaged DNA.

Here, we have undertaken two functional screens to identify negative regulators of p53 tumor suppressor activity. From each screen we isolated macrophage migration inhibitory factor (MIF).[1] Our observation that MIF, a proinflammatory cytokine released at the sites of inflammation, is capable of functionally inactivating p53, a tumor suppressor that normally functions to prevent proliferation of cells carrying genotoxic damage, may provide a mechanistic link between inflammation and cancer.

Materials and Methods

Construction of tet-GFP-p53 p53$^{-/-}$ Mouse Embryonic Fibroblast Cell Line. p53$^{-/-}$ mouse embryonic fibroblasts (MEFs; T. from

[1]*Abbreviations used in this paper:* FBS, fetal bovine serum; GSNO, *S*-nitrosoglutathione; MBP, maltose binding protein; MEF, mouse embryonic fibroblast; MIF, macrophage migration inhibitory factor; NO, nitric oxide; SNP, sodium nitroprusside.

GFP, green fluorescent protein

Jacks, Massachusetts Institute of Technology, Cambridge, MA) were sequentially infected with pWZL-Blast-rtta, a blasticidin selectable retroviral vector expressing the reverse transactivator of the tetracycline inducible system (22), and pBabe-puro-tet-GFP-p53-sin, a self-inactivating retrovirus expressing GFP-p53 fusion protein under the control of the tetracycline inducible promoter. Cells were drug selected, and a clone (TGP53-4) was isolated that showed observable GFP-p53 expression, and growth arrest of the cells after addition of 1 μg/ml doxycycline to the media.

Recombinant MIF. EcoRI and SalI sites were introduced immediately 5′ and 3′ to the open reading frame of human MIF by PCR, and this EcoRI-SalI fragment was cloned into EcoRI-XhoI sites of pMal-C2 (New England Biolabs). A maltose binding protein (MBP)–MIF fusion was expressed in BL21 *Escherichia coli* cells, affinity purified by amylose chromatography, and cleaved using factor Xa. MBP was removed after cleavage by amylose chromatography. Since MBP had no effect in any of the assays used, some experiments were performed using rMIF immediately after cleavage.

Bypass of p53-induced Growth Arrest. TGP53-4 cells were infected with a pHYGROMARX-I-derived provirus containing MIF cDNA or empty vector control. After hygromycin selection, cells were plated at ~5,000 cells/plate. 1 μg/ml doxycycline was added to induce p53 expression in appropriate plates. Media were replaced every 3 d containing fresh doxycycline where necessary. After 10 d, cells were fixed in 1% glutaraldehyde and stained with 0.25% crystal violet. For experiments using soluble rMIF, TGP53-4 cells were plated at ~10,000 cells/plate in the presence or absence of 150 ng/ml of rMIF added to the growth media. 24 h later, doxycycline was added to induce p53 expression. Media were replaced every 3 d containing fresh doxycycline and/or rMIF. After 9 d, cells were fixed and stained as above.

Elongation of Life Span of Primary Mouse Fibroblasts. MEFs were prepared from 14-d CD1 mouse embryos, and were repeatedly passaged. Where necessary, cells were infected in passage 2 with pMARXIV-p53as, pWZLneo-MIF, or control viruses, and selected by drug resistance for the selectable marker. One passage before the onset of senescence (usually around passage 4–5), cells were split and plated at ~300,000 cells/plate in the presence or absence of rMIF. Fresh tissue culture media (containing rMIF where appropriate) were replaced every 3 d. After 15–17 d, cells were fixed in 1% glutaraldehyde and stained with crystal violet. To determine cell concentration, crystal violet was resolubilized in 10% acetic acid and absorbance at 595 nm was analyzed using a Bio-Rad 550 microplate reader.

Apoptosis of Rat1/mycER Cells. Rat-1/mycER cells were infected with retroviruses expressing LacZ, MIF, or Bcl2 cDNAs. After drug selection, cells were plated onto acid-washed coverslips at low density and shifted to media containing 0.1% fetal bovine serum (FBS) plus 0.1 μM estradiol to induce apoptosis. After 24 h, cells were stained with 4 mg/ml Hoechst 33342 for 10 min, then washed and scored by fluorescent microscopy. Cells containing condensed or fragmented DNA cells were scored as apoptotic cells. At least 100 fields/slide were analyzed by two independent observers.

Apoptosis of RAW264.7 Macrophages. RAW264.7 macrophages were pretreated with varying concentrations of MIF for 24 h, and then treated with 0.25–1.0 mM sodium nitroprusside (SNP) or 0.5–1 mM *S*-nitrosoglutathione (GSNO) for 8 h to 2 d. Cells containing condensed or fragmented DNA after very brief fixing with paraformaldehyde and staining with Hoechst 33258 cells were scored as apoptotic cells.

Fluorescence Microscopy. TGP53-4 cells were split onto coverslips in the presence or absence of 150 ng/ml MIF. 24 h later, 1 μg/ml doxycycline was added to the media. 16 h after doxycycline addition, cells were washed in PBS and fixed in 2% paraformaldehyde, and GFP-p53 was visualized with a Zeiss Axiophot fluorescent microscope using a standard FITC filter set.

Western Blots. Cells were washed in PBS, harvested in PBS, centrifuged, and lysed. Equal amounts of total protein (30–300 μg) were heat-denatured, separated on a 10% SDS-polyacrylamide gel, and blotted to nitrocellulose. Blots were probed with antibodies that recognize p53 (DO-1, FL-393; Santa Cruz Biotechnology), MDM2 (SMP-14; Santa Cruz Biotechnology), BAX (BAX Δp21; Santa Cruz Biotechnology), or p21 (23) followed by a horseradish peroxidase–conjugated anti–mouse antibody, and detected using enhanced chemiluminescence.

Northern Blots. Total RNA was prepared from TGP53-4 cells after induction of GFP-p53. ~10 μg was separated in a 1% formaldehyde gel and blotted to Hybond-N$^+$ membranes. Blots were probed with random primed radiolabeled probes corresponding to the full-length coding sequence of mouse p21 and cyclin G. Radioactive signals were quantified using a Fuji FLA-200 phospho/fluorescent imager, and normalized to loaded RNA by quantification of fluorescence of ethidium bromide–stained ribosomal RNA bands on the RNA gel, or after blotting to the membrane.

Luciferase Assays. TGP53-4 cells were cotransfected with PG13, a plasmid which carries firefly luciferase under the control of three tandem copies of a p53-responsive consensus sequence, and pCDNA3-β-gal, a plasmid which carries β-galactosidase under the control of the CMV promoter. 1 d after transfection, cells were split, pooled, and replated at ~500,000 cells/plate. 150 ng/ml rMIF was added to half of the plates. The next day, 1 μg/ml doxycycline was added to the media to induce GFP-p53. At 0 and 10 h after induction, extracts were prepared, and luciferase and β-galactosidase activities were assayed using Promega kits. Luciferase reporter activities were normalized to β-galactosidase expression levels.

Results

MIF Isolated in Screens for Negative Regulators of p53 Activity. To identify novel regulators of p53 activity, we undertook a screen to identify genes that, when expressed at high level, were capable of bypassing p53-mediated growth arrest. A p53$^{-/-}$ MEF cell line was engineered to express a GFP-p53 fusion protein under the control of a tetracycline (doxycycline)-inducible promoter (22; TGP53-4 cell line). GFP-p53 fusion proteins are localized normally and can transactivate target genes (24; and data not shown). After addition of doxycycline to the media, the p53 fusion protein was induced, and cells became growth arrested and failed to form colonies.

We used the TGP53-4 cell line in a phenotype-based screen to identify negative regulators of p53 activity. These cells were infected with an A431 epidermoid carcinoma–derived cDNA library in a Moloney murine leukemia virus (MMLV)–based retroviral vector, pHYGROMARX-I (25). pHYGROMARX-I contains a bacterial origin of replication, zeocin resistance marker between the LTRs, and a loxP site in the 3′ LTR, which is duplicated upon integration, to facilitate provirus recovery by Cre-mediated exci-

sion after integration into the genome. LinX (25) ecotropic retrovirus producer cells were transiently transfected with this library, and after 3 d, supernatant was used to infect TGP53-4 cells. Approximately 4×10^6 cells were infected. After drug selection for the library vector, cells were split at varying dilutions, and 1 μg/ml doxycycline was added to the media to induce the GFP-p53 fusion protein. When necessary, cells were split again to improve colony discrimination. Cells that were no longer inhibited by p53 induction gave rise to colonies in the presence of doxycycline. These clones were infected with pBabe-puro-Cre, a Moloney murine leukemia virus–based virus that strongly expresses Cre recombinase to excise the provirus. Proviruses containing cDNAs from positive clones were recovered by HIRT extraction.

Proviruses were recovered from a total of 50 positive colonies. Nucleotide sequencing and database analysis revealed that cDNAs recovered from five different colonies encoded the same protein, human MIF, a cytokine that was shown previously to exert both local and systemic proinflammatory activities (26). All cDNAs encoding MIF were full length and in the sense orientation. The complete upstream regions were sequenced from three of these recovered cDNAs. Two differed in the precise 5′ terminus, indicating that they were derived from independent clones.

A cDNA-encoding MIF was also independently isolated in a similar phenotype-based screen to identify negative regulators of myc-dependent apoptosis in rat fibroblasts. Rat-1 fibroblasts expressing a c-myc–estrogen receptor fusion protein (Rat1/mycER) were infected with pools of a cDNA library prepared from Rat-1/mycER cells committed to apoptosis in pHygroMarxII. After drug selection, cells were induced to undergo apoptosis by shifting to low serum media (DMEM + 0.1% FBS) plus 0.1 μM estradiol (to induce c-myc activity) for 3 d, followed by 2 d of serum starvation without estradiol. Cells that were protected from apoptosis were recovered in media containing 10% FBS. Rescued cells were subjected to three additional cycles of apoptotic induction. Proviruses were recovered from apoptosis-resistant cells by Cre-mediated excision of genomic DNA line (27). Since this screen was carried out in a cell line expressing wild-type p53, and myc-driven apoptosis is largely p53 dependent (28), inhibitors of p53 function were expected to be recovered from this screen.

MIF Treatment Bypasses p53-mediated Growth Arrest. To confirm that MIF was capable of bypassing p53-mediated growth arrest, a provirus containing MIF or a control provirus was transduced into TGP53-4 cells. Doxycycline was added to induce p53 expression. Numerous colonies formed on plates containing MIF-expressing cells, but few or no colonies formed on plates containing control cells (Fig. 1 A).

Since MIF was originally identified as an extracellular cytokine, we tested whether MIF protein could overcome p53-mediated growth arrest upon addition as a recombinant protein to the culture medium. MIF protein was produced as an MBP fusion protein, and cleaved to separate MIF from MBP (Fig. 1 B). TGP53-4 cells were grown in

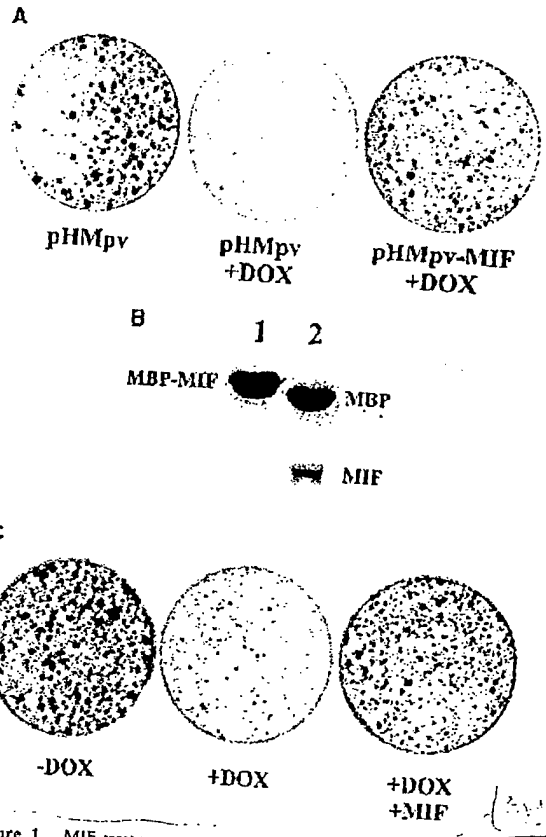

Figure 1. MIF treatment overcomes p53-induced growth arrest. (A) Expression of MIF bypasses p53-induced growth arrest and allows colony formation in a tetracycline-inducible GFP-p53 cell line. pH Mpv, HYGROMARXI-based provirus; HMpv-MIF, HYGROMARXI-based provirus expressing human MIF; DOX, 1 μg/ml doxycycline. (B) Recombinantly produced MBP-MIF before (lane 1) and after cleavage (lane 2). No contaminating bands were observed in Coomassie blue or Sypro orange–stained gels. (C) Addition of 150 ng/ml soluble rMIF bypasses p53-induced growth arrest of a tetracycline-inducible GFP-p53 cell line.

the presence or absence of recombinantly produced MIF (rMIF) and doxycycline. Colony formation was observed in the absence of doxycycline, or in the presence of doxycycline, and rMIF, but not in the presence of doxycycline alone. Therefore, MIF was capable of bypassing p53-mediated growth arrest when added as a soluble factor (Fig. 1 C).

MIF Treatment Suppresses p53-dependent Transcriptional Activation. p53 might be inactivated by altering its subcellular localization, by decreasing protein levels, or by suppressing its ability to function as a transcriptional activator. Since GFP-p53 can be visualized directly in cells and shows normal subcellular localization, we analyzed whether p53 showed altered subcellular localization in the presence of MIF. No obvious difference in the subcellular localization of GFP-p53 was observed; p53 showed nuclear localization irrespective of MIF treatment (Fig. 2 A). p53 can also be regulated by altering protein abundance; however, p53 protein levels were not reduced after MIF treatment (Fig. 2 B).

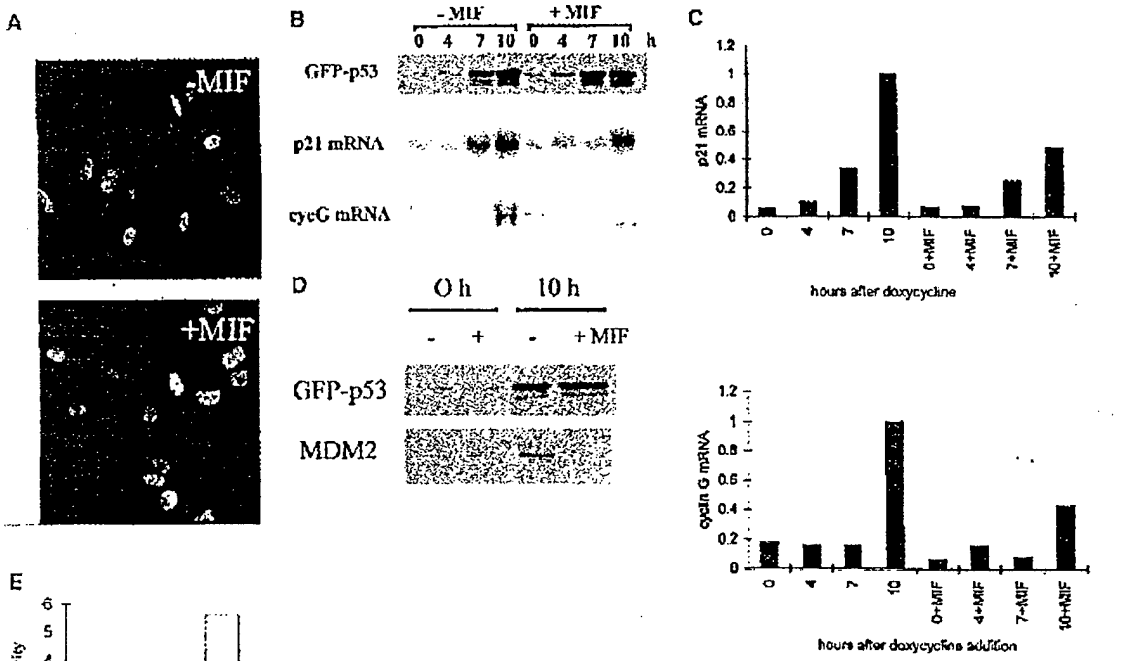

Figure 2. MIF suppresses the transcriptional activity of p53. (A) Subcellular localization of GFP-p53 in the presence or absence of MIF. GFP-p53 shows normal nuclear localization in each case. (B and C) Levels of GFP-p53 protein, and p21 and cyclin G mRNA after induction of GFP-p53 in the presence or absence of MIF. Data shown in C are averages from three independent experiments. (D) Levels of GFP-p53 and MDM2 protein before or 10 h after induction of GFP-p53 in the presence or absence of MIF. (E) p53-responsive luciferase reporter gene expression in the presence or absence of MIF after p53 induction. MIF treatment suppresses reporter gene expression. Data shown are averages from four independent experiments.

p53 primarily functions via its ability to transactivate gene expression. Therefore, we tested whether MIF treatment interfered with this activity. After induction of p53, RNA was prepared from TGP53-4 cells grown in the presence or absence of MIF. The abundance of two p53 transcriptional targets, p21 (29–31) and cyclin G (32), was assessed by Northern blot (Fig. 2 B). Levels of p21 and cyclin G in MIF-treated cells were decreased to ~50 and 40% of control levels (Fig. 2 C). In addition, p53-dependent induction of MDM2, another p53 target which acts in a feedback loop to negatively regulate levels of p53 (33, 34) was decreased in MIF-treated cells (Fig. 2 D).

The effect of MIF treatment on the activity of a p53-dependent reporter was also assayed. TGP53-4 cells were transfected PG13-luc, a plasmid which carries firefly luciferase under the control of tandem copies of a p53-responsive consensus sequence (35), in the presence and absence of MIF, and luciferase activity was assayed after induction of GFP-p53. Treatment with rMIF suppressed p53-dependent luciferase expression (Fig. 2 E). Considered together, these data suggest that MIF treatment bypassed p53-mediated growth arrest by suppressing p53-dependent transcriptional activation.

MIF Treatment Suppresses p53-dependent Apoptosis. In addition to its ability to induce growth arrest, p53 functions to induce apoptosis in response to cellular stress in susceptible cells (5, 7, 8). As described above, we isolated a cDNA-encoding MIF in a screen designed to identify inhibitors of myc-dependent apoptosis, a process which is largely p53-dependent. To formally confirm that MIF expression could suppress this phenotype, Rat-1/mycER cells were infected with an MIF-expressing virus and control viruses, and apoptosis was induced by serum starvation and estradiol treatment. Cells that expressed MIF were partially protected from apoptosis under these conditions, though not as efficiently as cells that expressed Bcl2 (Fig. 3 A).

Since MIF regulates numerous functions of macrophages in in vitro assays and in vivo, we also tested whether MIF treatment was capable of inhibiting apoptosis in macrophages. After activation, macrophages release nitric oxide (NO) as part of their antimicrobial repertoire. However, high levels of NO can, in turn, cause macrophage apoptosis. For example, apoptosis is induced by treatment of RAW264.7 macrophages with cytokines that induce endogenous production of NO, or with chemical releasers of NO. Apoptosis is associated with induction of p53 and is inhibited by expression of antisense p53 constructs, indicating that NO-induced macrophage apoptosis is p53 depen-

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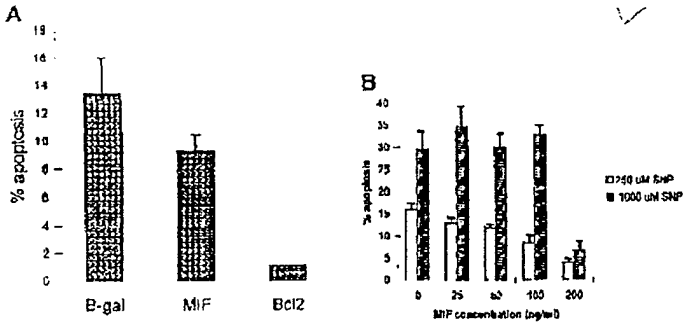

Figure 3. MIF treatment overcomes p53-dependent apoptosis in fibroblasts and macrophages. (A) Apoptosis in Rat1/mycER cells. Rat-1/mycER cells expressing LacZ, MIF, or Bcl2 cDNAs were shifted to media containing 0.1% FBS plus 0.1 μM estradiol to induce apoptosis. After 24 h, cells were stained with Hoechst 33342 and scored. Cells containing condensed or fragmented DNA cells were scored as apoptotic cells. (B) RAW264.7 macrophages were pretreated with varying concentrations of MIF for 24 h, and then treated with 250 μM or 1 mM SNP. Apoptotic nuclei were scored after 2 d. (C) RAW264.7 macrophages were pretreated with MIF for 16 h, treated with SNP or GSNO for 8 h, and apoptotic cells were scored. 1, 0.5 mM SNP; 2, 1.0 mM SNP; 3, 0.5 mM GSNO; 4, 1.0 mM GSNO; 5, no treatment.

dent (36, 37). To test whether MIF treatment was capable of suppressing NO-induced apoptosis, we treated RAW264.7 macrophages with NO-releasers, SNP, or GSNO, in the presence of various concentrations of rMIF. MIF treatment suppressed NO-induced apoptosis in a dose-dependent manner (Fig. 3, B and C).

MIF Treatment Extends the Life Span of Primary Murine Fibroblasts. p53 also plays a role in controlling the onset of cellular senescence (12–14). Normal primary mouse fibroblasts are capable of a finite number of divisions in culture, and ultimately arrest with a senescent morphology (11). Loss of p53 allows primary mouse cells to extend their division potential. Thus, in a colony formation assay, cells lacking p53 are capable of forming colonies at passages at which wild-type cells are not. Therefore, we tested whether MIF was capable of elongating the potential life span of primary MEFs. At one passage before the onset of senescence (passage 4–5), primary MEFs were plated in the presence or absence of rMIF. After 15 d, numerous colonies had formed on plates treated with MIF, whereas none were observed in the absence of MIF. This indicated that MIF treatment, like loss of p53, was capable of inducing elongated life span (Fig. 4 A). Colony formation occurred at a frequency of $\sim 10^{-4}$ colonies/cell (the frequency of colony formation observed with cells expressing an antisense or dominant negative p53 under identical conditions is $2–3 \times 10^{-4}$ and $1–3 \times 10^{-3}$ with fibroblasts prepared from a $p53^{-/-}$ mouse; Carnero, A., and D. Beach, unpublished). To determine the concentration of MIF that was optimal for colony-forming activity, we repeated the experiment in the presence of 0–600 ng/ml rMIF. Elongation of life span was dose dependent, with 150 ng/ml giving the most pronounced effect (Fig. 4 B).

Biological Activity of MIF Correlates with Its Ability to Suppress p53-responsive Gene Expression in Extending Life Span of Primary MEFs. Since MIF treatment does not completely negate p53-mediated gene expression, we sought to test whether the ability of MIF to induce a p53-related biological activity correlated with the relative suppression of p53-mediated gene expression. Primary MEFs were infected with a virus expressing MIF, an antisense construct directed against p53 or control virus in passage 2. Infected cells were selected for drug resistance, cultured, and plated on duplicate plates in passage 5. At the same time, MEFs in passage 5 were plated in the presence or absence of rMIF. 15 d after plating, one of each duplicate plate was fixed and stained with crystal violet (Fig. 5 A). Protein extracts were prepared from the other duplicate plate, and the levels of

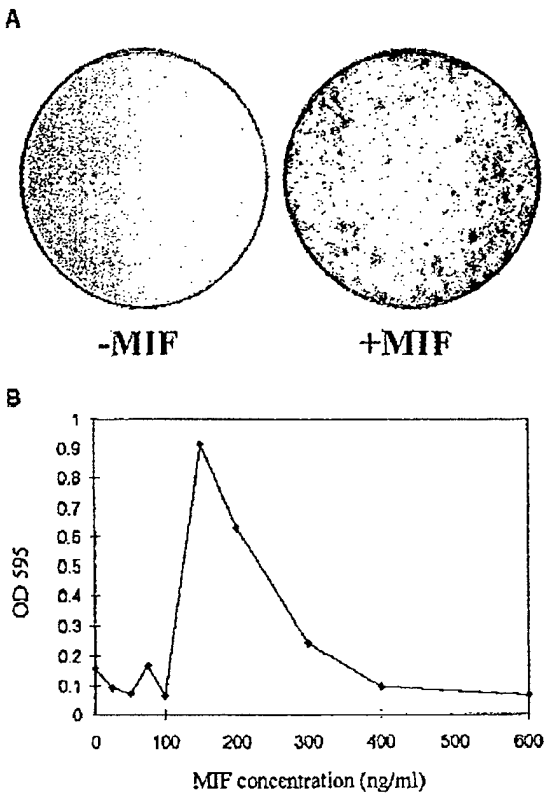

Figure 4. (A) Primary MEFs show extended life span in the presence of 200 ng/ml rMIF. Cells one passage before senescence were plated in the presence and absence of MIF, and stained after 15 d. Numerous colonies were formed only in the presence of MIF. (B) Dose dependency of MIF treatment in inducing extended life span. Primary cells, as in A, were grown in the presence of varying concentrations of MIF. After 17 d, cells were crystal violet stained, and washed. Resolubilized crystal violet was assayed as a measure of cell density.

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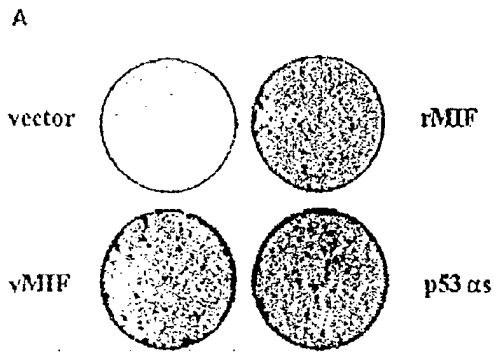

Figure 5. MIF biological activity correlates with suppression of p53-mediated target gene expression. Primary MEFs expressing MIF (vMIF), an antisense directed against p53 (p53αs), control vector (vector), or treated with rMIF were plated in passage 5. 15 d after plating cells were (A) fixed and stained with crystal violet or (B) lysed, and extracts were probed for p53, Bax, or p21 expression by Western blot.

p53 and two p53 targets, p21 and bax (38), were assayed by Western blot (Fig. 5 B). In each case, the number of colonies observed roughly correlated with the relative suppression of p53 target gene expression, consistent with the hypothesis that suppression of p53 activity is largely responsible for this MIF-induced biological activity.

Discussion

We have demonstrated that MIF treatment was capable of overcoming p53 activity in three distinct biological assays. The ability of a secreted factor to overcome a growth-inhibitory pathway that has been associated with cellular mortality and with the response of cells to genotoxic stress may have an important physiological role. At sites of inflammation, MIF is released from T cells and from macrophages (26). High local concentrations of MIF contribute to T cell activation and enhance the antimicrobial activity of macrophages (39, 40). When activated, macrophages release NO and other oxide radicals (41). However, NO can also induce macrophage apoptosis. Since MIF can partially negate the p53 response and can protect macrophages from NO-induced apoptosis, this factor may normally act to protect macrophages from the destructive machinery they use to kill invading organisms.

Inflammatory loci are characterized by high rates of cell death and compensatory proliferation in adjacent cells (42). At the same time, upregulation of p53 is often observed (43, 44). Overcoming p53 activity through MIF action may help to limit the damage response, and therefore to limit the loss of host cells and to permit local cell proliferation for tissue repair. After cessation of the inflammatory state, local levels of MIF decrease, allowing restoration of the normal damage response.

However, chronic bypass of p53 function by MIF could contribute to the development of tumors. Loss of p53 function is one of the most common events in human cancer. Cells that lack p53 function have enhanced proliferative potential and display extended life span. In addition, cells lacking functional p53 are deficient in responding to chromosome damage (9, 10). During inflammation, release of highly reactive oxidants by activated phagocytes has been implicated in the induction of DNA damage in neighboring cells (20, 21). In the chronic presence of MIF, cells with attenuated p53 function might continue to proliferate in the presence of DNA damage, and eventually accumulate multiple oncogenic mutations.

Several chronic inflammatory conditions are strongly associated with eventual tumor formation (18, 19). For example, ulcerative colitis or Crohn's disease is associated with the eventual development of bowel cancer, whereas reflux esophagitis or Barrett's syndrome has been linked to the development of esophageal cancer. Schistosomiasis infection predisposes to the development of urinary bladder cancer, and long term *Helicobacter pylori* infection has been implicated in the development of gastric cancer. In some cases of *H. pylori* infection, ablation of the infectious agent is correlated with reversal of the inflammatory state and with regression of the associated tumor. This suggests that, in this model, at least one tumorigenic event requires continued presence of the inflammatory state, and is reversible (45). The observation that MIF can interfere with p53 function may provide insight into the mechanisms by which certain chronic inflammatory conditions predispose individuals to tumor formation.

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