

AD\_\_\_\_\_

Award Number: DAMD17-96-1-6053

TITLE: Complementation Screening in Mammalian Cells: Application  
to Cell Immortalization

PRINCIPAL INVESTIGATOR: Gregory J. Hannon, Ph.D.

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory  
Cold Spring Harbor, New York 11724

REPORT DATE: October 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20010322 156

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

**1. AGENCY USE ONLY (Leave blank)****2. REPORT DATE**  
October 2000**3. REPORT TYPE AND DATES COVERED**  
Final (1 Sep 96 - 1 Sep 00)**4. TITLE AND SUBTITLE**

Complementation Screening in Mammalian Cells: Application to Cell Immortalization

**5. FUNDING NUMBERS**

DAMD17-96-1-6053

**6. AUTHOR(S)**

Gregory J. Hannon, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**Cold Spring Harbor Laboratory  
Cold Spring Harbor, New York 11724**8. PERFORMING ORGANIZATION REPORT NUMBER****E-MAIL:**

hannon@cshl.org

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012**10. SPONSORING / MONITORING AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES**

Report contains color photos

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for public release; distribution unlimited

**12b. DISTRIBUTION CODE****13. ABSTRACT (Maximum 200 Words)**

The transformation of a normal cell into a cancer cell occurs through a series of genetic and epigenetic alterations that release a cell from controls that restrain its proliferation. The purpose of the research supported by this new investigator award was, broadly, two-fold. The primary goal was to increase our understanding of the transformation process by focussing on one aspect of neoplastic cells, their ability to proliferate indefinitely. The secondary goal – which served as a means to the principal end – was to develop a vector system that would allow complementation screening in cultured mammalian cells. During the tenure of this award, both goals were largely achieved. We have developed a series of retroviral vectors that allow the introduction of complex libraries into a broad range of cell types. We have validated these vectors for complementation screens and have pursued several findings that are cancer-relevant. Finally, we have taken a step toward understanding the ability of tumor cells to proliferate indefinitely by linking a commonly activated oncogene, c-myc, to the activity of the telomerase enzyme.

**14. SUBJECT TERMS**

Breast Cancer

**15. NUMBER OF PAGES**

59

**16. PRICE CODE****17. SECURITY CLASSIFICATION OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION OF ABSTRACT**

Unclassified

**20. LIMITATION OF ABSTRACT**

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

## Table of Contents

|                                   |       |
|-----------------------------------|-------|
| Cover.....                        | 1     |
| SF 298.....                       | 2     |
| Table of Contents.....            | 3     |
| Introduction.....                 | 4     |
| Body.....                         | 5-8   |
| Key Research Accomplishments..... | 8     |
| Reportable Outcomes.....          | 8-9   |
| Conclusions.....                  | 10    |
| References.....                   | 11    |
| Appendices List.....              | 12    |
| Bibliography & Personnel.....     | 13    |
| Current contact information.....  | 14    |
| Appendix.....                     | 15-48 |

## Introduction

The transformation of a normal cell into a cancer cell occurs through a series of genetic and epigenetic alterations that release a cell from controls that restrain its proliferation. The longstanding goal of my laboratory has been to contribute to the developing understanding of the processes that are keystones of the transformation process. Accumulating data has suggested that there are several key landmarks on the path toward neoplasia. Among these are inactivation of key tumor suppression pathways such as those which govern genome integrity (e.g. p53) and those which regulate the decision to enter the cell division cycle (e.g. Rb). In addition to these changes, cells must acquire the ability to proliferate beyond their normal replicative lifespan<sup>4</sup>.

The purpose of the research supported by this new investigator award was, broadly, two-fold. The primary goal was to increase our understanding of the transformation process by focussing on one property of neoplastic cells, their ability to proliferate indefinitely. The secondary goal – which served as a means to the principal end – was to develop a vector system that would allow complementation screening in cultured mammalian cells. During the tenure of this award, both goals were largely achieved. We have developed a series of retroviral vectors that allow the introduction of complex libraries into a broad range of cell types. We have validated these vectors for complementation screens and have pursued several findings that are cancer-relevant. Finally, we have taken a step toward understanding the ability of tumor cells to proliferate indefinitely by linking a commonly activated oncogene, c-myc, to the activity of the telomerase enzyme.

## Body

*Statement of work Technical objective 1 : Development of retroviral vectors that are useful for complementation screening in mammalian cells.*

### Tasks 1 and 2 – development and testing of the retroviral system

One of the goals of this New Investigator award was the development of retroviral vector tools that were useful for complementation screening in mammalian cells. The hypothesis that underlay this goal was that we could identify genes that are involved in specific biological pathways by functional criteria alone. Modeling our approach after those that have been successful in yeast, we developed the MaRX system. This gene transfer system is based on recombinant, replication-deficient retroviral vectors. This design was chosen for a variety of reasons. First, retroviral vectors had been well characterized and had been shown to allow efficient gene transfer into a broad range of cell types. Second, we felt that we could modify with relative ease these vectors to meet our criteria for complementation screening. As is always the case in science, construction and testing of the vectors took longer than was originally anticipated. In addition, we were forced to create both amphotropic and ecotropic packaging cell lines because those that were available at the time at which we started this work were unstable. These cells (LinX cells) are now widely used for retrovirus production. Thus, we have succeeded in developing a workable system that is now being used by many researchers. The philosophy of the system design and the details of its use are described in several publications that are provided as appendices 1 to 7.

### Task 3 – preparation of cDNA libraries in the retroviral vectors

Over the four years of support, we have prepared approximately 25 cDNA libraries in the MaRX vector system. All have complexities exceeding 2 million primary clones. Included are libraries from NIH-3T3 cells, mouse embryo (mixed stage), mixed human breast carcinoma material and numerous transformed cell lines. Many have been prepared in both the sense and antisense orientation. The use of some of these libraries is described in publications that are included as appendices 1 to 6. Furthermore, we have made these libraries available to numerous investigators who are pursuing aspects of the transformation process in breast and other cancer models.

*Technical Objectives 2 and 3 – A search for genes that activate telomerase and thus may immortalize primary cells.*

### Task 4. Testing of cell lines for use in complementation screens.

During the term of the support, we have validated the use of our vector systems in a variety of cell types. A subset of these are described in appendices 1 to 7. In short, the only cell types in which we have failed to obtain successful transduction are

certain primary, undifferentiated cell types that silence retroviral gene expression by methylation of the LTR promoter. For example, MaRX vectors perform poorly in primary murine B-cells. We are currently designing a vector series that uses the Mouse Embryonic Stem Cell Virus LTR to avoid these problems.

Task 5 – Screening of libraries for the ability to induce telomerase in primary human mammary epithelial cells.

We screened ~500 pools of 10,000 genes each for the ability to induce telomerase activity in primary cells. From this effort, we had a few putative positives that were subsequently discovered to be false positives.

Task 6 – Screening of cDNA libraries for the ability to induce M1 bypass.

This effort was abandoned since conditionally immortal cells showed a reversion frequency that was far higher than was acceptable for screening. We are presently pursuing screens for senescence bypass in primary mouse embryo fibroblasts which show a much tighter phenotype. This is part of an ongoing collaboration with Amancio Carnero and David Beach at University College in London. Our efforts to validate the system using p53 antisense as a positive control are described in a publication that is included as Appendix 6.

Task 7 – Subdivision and secondary screening of pools that gave active telomerase.

In total, approximately 20 pools were screened in this manner and were found to be false-positives.

Task 8 – see Task 6.

Task 9 – Biological function of clones that induce telomerase in HMEC cells.

During the course of our screening for clones that induce telomerase in HMEC cells, we carried out a survey of known oncogenes for the ability to induce telomerase. We found that ectopic expression of c-myc could induce telomerase in normal fibroblasts and in normal HMEC to a degree that approximated that seen in similarly-derived tumor cells. Both c-myc and telomerase could immortalize primary human fibroblasts and so-called primary human mammary epithelial cells, strongly implying that one function of c-myc during tumor formation might be to defeat controls that normal limit proliferative capacity. This represents one of very few definitive links between myc and aspects of neoplastic transformation. Subsequent studies by other laboratories have shown that myc activates directly the promoter of the gene that encodes the limiting subunit of the telomerase enzyme (1, 2). Furthermore, in vivo, there is a high-degree of correlation between myc expression and telomerase activation in both normal and neoplastic tissues. This, myc may regulate telomerase during the execution of scheduled proliferative programs in normal cells (e.g. in expansion of B and T cells), and this property of myc might be exploited during the transformation

process. A publication describing these findings is included as Appendix 1. Additional relevant information regarding the use of telomerase as a tool to immortalize cells for therapeutic purposes is included as Appendix 7.

Task 10 – see Task 6.

Task 11 – see Task 6.

### *Discussion*

The broad goals of this application were to develop a set of tools that would enhance our ability to dissect biological problems using tissue culture cells as a model system and to apply that system to enhance our understanding of cellular mortality control. We originally divided the mortality control problem into two distinct steps, M1 and M2. However, it is now clear both through our work (as supported by this award) and that of others, that these two barriers to indefinite proliferation are really responses to the same underlying signal, telomere depletion. For this reasons, we have not vigorously pursued the original goal of identifying genes that could bypass M1 since the property that is probably most relevant to tumor cells is the immortality that is achieved by telomerase activation. Our investigations of this aspect of mortality control led to the discovery that an oncogene that is affected in a large percentage of breast and other cancers, c-myc, regulates both telomerase expression and cellular lifespan (i.e. proliferative capacity). However, telomere depletion is almost certainly irrelevant in mouse, and we have turned our attention somewhat to the mechanisms of mortality control in primary MEF cells. This is likely to be relevant to human tumorigenesis since epithelial cells (such as HMEC) encounter an early proliferative block that is telomere-independent and that may be overcome by inactivation of the p16 tumor suppressor (3).

Our ability to manipulate gene expression in primary cells grew out of our efforts toward the other goal of the original proposal, the development of tools for complementation screening in tissue culture cells. Our focus was the development of a retroviral vector system that allowed not only efficient introduction of complex populations of genes into cultured cells but also the ability to recover in an active configuration either single genes or gene libraries. The principles underlying the use of the MaRX system are described in a number of publications that have been included as appendices.

Not only did the development of these tools enhance our ability to manipulate normal cells but these efforts also led to a number of insights that are relevant to our understanding of breast and other cancers. One common property of transformed cells is their insensitivity to growth inhibitory cytokines such as TGF- $\beta$ . Using a genetic screen, we identified cDNAs that could bypass TGF- $\beta$ -mediated growth arrest in fibroblasts and in HMEC cells. Among the insights provided by this work was support for the idea that MDM2 could contribute to transformation independently of its ability to nullify the p53 pathway. This helps to provide an explanation for the lack of a negative

correlation between p53 mutations and MDM2 overexpression in breast and other cancers.

Oncogenes such as myc and E1a may promote cell proliferation but these proteins also predispose cells to programmed cell death. This requires that transformed cells develop means to counter these pro-apoptotic effects. A screen for genes that could protect from myc-induced apoptosis identified Twist, a member of the bHLH family of transcription factors, along with a number of other genes that are currently being analyzed. Although enhanced expression of Twist does not seem to contribute to breast cancer, it is a common feature of Rhabdomyosarcomas.

While we failed to complete a number of the specific tasks set out in the original statement of work, I feel that we succeeded in meeting all of the broad goals set out in the application.

### **Key Research Accomplishments**

- Development of a retroviral gene transfer system for complementation screening (see appendix 3)
- Demonstration that this system could be used to address cancer relevant biological questions (see appendices 2,4,5,6)
- Demonstration that MDM-2 may contribute to breast cancer through effects on the Rb pathway (see appendix 2)
- Identification of genes that can bypass p53-mediated growth arrest, including an extracellular factor that may help to explain the link between chronic inflammation and tumorigenesis. (see appendix 5)
- Identification of Twist as an antagonist of myc-induced apoptosis and as a potential oncogene (see appendix 4)
- Validation of systems in which to examine the genetics of senescence in mouse cells (see appendix 6)
- Demonstration that telomerase activation can immortalize primary cells (see appendix 1)
- Demonstration that c-myc regulates telomerase and cellular mortality (see appendix 1)
- Demonstration that immortalization by telomerase is not a neutral event. Instead, activation of myc is positively selected during outgrowth (Appendix 7).

### **Reportable outcomes**

*Manuscripts, Abstracts, Presentations*

Wang, J., Xie, L.-Y., Allan, S., Beach, D. and Hannon G.J. Myc activates telomerase  
Genes & Dev. **12**: 1769-1774.

Sun, P., Dong, P., Dai, K., Hannon, G.J. and Beach. P53-independent role of MDM2 in TGF- $\beta$ 1 resistance. *Science* **282**: 2270-2272.

Hannon, G.J., Sun, P., Carnero, A., Xie, L.-Y., Maestro, R., Conklin, D. and Beach. MaRX: An approach to genetics in mammalian cells. *Science* **283**: 1129-1130.

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

Hudson, J., Shoaibi, M., Maestro, R., Carnero, A., Hannon, G.J., and Beach, D. A proinflammatory cytokine inhibits p53 tumor suppressor activity. *J. Exp. Med.* **190**: 1375-1382.

Carnero, A., Hudson, J., Hannon, G.J. and Beach, D. Loss-of-functiona genetics in mammalian cells: the p53 tummor suppressor model. *Nuc. Acids Res.* **28**: 2234-2241.

Wang, J., Hannon, G.J. and Beach, D. Risky immortalization by telomerase. *Nature* **405**: 755-756.

#### *Patents*

|      |                      |                                                          |
|------|----------------------|----------------------------------------------------------|
| 1998 | Patent No. 6,025,192 | Modified Retroviral Vectors                              |
| 1999 | Patent No. 5,962,316 | Cell-Cycle Regulatory Proteins, and Uses Related Thereto |

#### *Reagents developed during the course of the work*

MaRX vector system  
LinX packaging cells

#### *Funding applied for based upon this work*

##### Funded

|                                                                                    |               |
|------------------------------------------------------------------------------------|---------------|
| DAMD17-00-1-0206 (Hannon)                                                          | 6/1/00-7/1/04 |
| Dept. of the Army - CDA                                                            | \$75,000      |
| Synthetic Lethality in Breast Cancer Cells: Genes Required for Tumor Cell Survival |               |

|                                                                                    |               |
|------------------------------------------------------------------------------------|---------------|
| DAMD17-00-1-0207 (Hannon)                                                          | 6/1/00-7/1/04 |
| Dept. of the Army - CDA                                                            | \$54,630      |
| Synthetic Lethality in Breast Cancer Cells: Genes Required for Tumor Cell Survival |               |

##### Pending

Department of the Army Postdoctoral Traineeship Award Proposal  
"A Molecular Basis of Genomic Instability in Prostate Cancer: Regulation of the Centrosome Duplication Cycle"

Department of the Army Idea Development Award  
"A Molecular Basis of Genomic Instability in Prostate Cancer: Regulation of the Centrosome Duplication Cycle".

## Conclusions

This New Investigator Award provided critical support in our efforts toward understanding the process of neoplastic transformation and tumorigenesis in human breast cancer. By most measures, the research supported under this award has produced a number of significant findings. A total of 7 publications in major journals (including Science and Genes and Development) resulted from the funded research. More importantly, we made seminal contributions to the understanding of cellular mortality control, forging the first link between telomerase activity and a *bona fide* human oncogene. Finally, we created a set of reagents, including retroviral vectors and packaging cells, that are now being widely used for cell engineering and for the more complex process of complementation screening in tissue culture cells. This support has led us in a number of new directions which form the subject of additional application to the U.S. Army Cancer Research programs that are either pending or that have been funded. Finally, our work has, in part, formed the basis of recent efforts – begun by the Weinberg laboratory and being pursued in numerous laboratories including my own – to create human tumor cells via defined genetic alterations. Our hope is that such studies will form the basis of an enhanced understanding of the genetics of human tumorigenesis.

## REFERENCES

1. Wu, K. J., Grandori, C., Amacker, M., Simon-Vermot, N., Polack, A., Lingner, J. & Dalla-Favera, R. (1999) *Nat Genet* **21**, 220-4.
2. Eberhardy, S. R., D'Cunha, C. A. & Farnham, P. J. (2000) *J Biol Chem*.
3. Kiyono, T., Foster, S. A., Koop, J. I., McDougall, J. K., Galloway, D. A. & Klingelhutz, A. J. (1998) *Nature* **396**, 84-8.
4. Hahn, W.C., Counter, C. M., Lundberg, A. S., Beijersbergen R.L., Brooks M.W. and Weinberg R.A. (1999) *Nature* **400**, 464-468.

## APPENDIX

- #1 Wang, J., Xie, L.-Y., Allan, S., Beach, D. and Hannon G.J. Myc activates telomerase *Genes & Dev.* **12**: 1769-1774.
- #2 Sun, P., Dong, P., Dai, K., Hannon, G.J. and Beach. P53-independent role of MDM2 in TGF- $\beta$ 1 resistance. *Science* **282**: 2270-2272.
- #3 Hannon, G.J., Sun, P., Carnero, A., Xie, L.-Y., Maestro, R., Conklin, D. and Beach. MaRX: An approach to genetics in mammalian cells. *Science* **283**: 1129-1130.
- #4 Maestro, R., Dei Tos, A., Hamamori, Y., Krasnokutsky, S., Sartorelli, V., Kedes, L., Doglioni, C., Beach, D. and Hannon, G.J. *twist* is a potential oncogene that inhibits apoptosis. *Genes & Dev* **13**: 2207-2217.
- #5 Hudson, J., Shoaibi, M., Maestro, R., Carnero, A., Hannon, G.J., and Beach, D. A proinflammatory cytokine inhibits p53 tumor suppressor activity. *J. Exp. Med.* **190**: 1375-1382.
- #6 Carnero, A., Hudson, J., Hannon, G.J. and Beach, D. Loss-of-functiona genetics in mammalian cells: the p53 tummor suppressor model. *Nuc. Acids Res.* **28**: 2234-2241.
- #7 Wang, J., Hannon, G.J. and Beach, D. Risky immortalization by telomerase. *Nature* **405**: 755-756.
- #8 Curriculum Vitae - G. J. Hannon

## BIBLIOGRAPHY

- Wang, J., Xie, L.-Y., Allan, S., Beach, D. and Hannon G.J. Myc activates telomerase Genes & Dev. **12**: 1769-1774.
- Sun, P., Dong, P., Dai, K., Hannon, G.J. and Beach. P53-independent role of MDM2 in TGF- $\beta$ 1 resistance. Science **282**: 2270-2272.
- Hannon, G.J., Sun, P., Carnero, A., Xie, L.-Y., Maestros, R., Conklin, D. and Beach. MaRX: An approach to genetics in mammalian cells. Science **283**: 1129-1130.
- Maestro, R., Dei Tos, A., Hamamori, Y., Krasnokutsky, S., Sartorelli, V., Kedes, L., Doglioni, C., Beach, D. and Hannon, G.J. *twist* is a potential oncogene that inhibits apoptosis. Genes & Dev **13**: 2207-2217.
- Hudson, J., Shoaibi, M., Maestro, R., Carnero, A., Hannon, G.J., and Beach, D. A proinflammatory cytokine inhibits p53 tumor suppressor activity. J. Exp. Med. **190**: 1375-1382.
- Carnero, A., Hudson, J., Hannon, G.J. and Beach, D. Loss-of-functiona genetics in mammalian cells: the p53 tummor suppressor model. Nuc. Acids Res. **28**: 2234-2241.
- Wang, J., Hannon, G.J. and Beach, D. Risky immortalization by telomerase. Nature **405**: 755-756.

## LIST OF PERSONNEL WHO RECEIVED PAY FROM THE RESEARCH EFFORT

|                   |                  |
|-------------------|------------------|
| Gregory J. Hannon | P.I.             |
| Ping Dong         | Lab Technician   |
| Sabrina Boettcher | Lab Technician   |
| Emily Bernstein   | Graduate Student |
| Yvette Seger      | Graduate Student |

Current Address Fax and e-mail

Gregory J. Hannon  
Cold Spring Harbor Laboratory  
1 Bungtown Road  
Cold Spring Harbor, NY 11724

Tel: 516-367-8889  
Fax: 516-367-8874  
E-mail: [hannon@cshl.org](mailto:hannon@cshl.org)

## RESEARCH COMMUNICATION

**Myc activates telomerase**Jing Wang,<sup>1</sup> Lin Ying Xie,<sup>1</sup> Susan Allan,<sup>1</sup>  
David Beach,<sup>2</sup> and Gregory J. Hannon<sup>1,3</sup><sup>1</sup>Cold Spring Harbor Laboratory (CSHL), Cold Spring Harbor, New York 11724 USA; <sup>2</sup>The Institute of Child Health, London WC1N 1EH, UK

**Telomere maintenance has been proposed as an essential prerequisite to human tumor development. The telomerase enzyme is itself a marker for tumor cells, but the genetic alterations that activate the enzyme during neoplastic transformation have remained a mystery. Here, we show that Myc induces telomerase in both normal human mammary epithelial cells (HMECs) and normal human diploid fibroblasts. Myc increases expression of hEST2 (hTERT/TP2), the limiting subunit of telomerase, and both Myc and hEST2 can extend the life span of HMECs. The ability of Myc to activate telomerase may contribute to its ability to promote tumor formation.**

Received April 3, 1998; revised version accepted April 27, 1998.

Telomerase activity is largely absent from somatic cells in vivo and from normal human cells in culture (Counter et al. 1992). As these cells proliferate, telomeric repeats are progressively lost as a result of incomplete replication of chromosome ends during each division cycle (Watson 1972; Olovnikov 1973; Harley et al. 1990; Hastie et al. 1990). Telomere shortening has been proposed as the mitotic clock that marks the progress of a cell toward the end of its replicative life span. According to this model, erosion of chromosome ends triggers cellular senescence (Harley et al. 1990; for review, see Harley 1991). Bypass of senescence can be accomplished by negation of tumor suppressor pathways (e.g., p53 and Rb/p16). This allows continued proliferation (extended life span) that is accompanied by further telomere loss (Counter et al. 1992). Indefinite proliferation in the absence of a telomere maintenance strategy would eventually result in a complete loss of telomeres and in destabilization of chromosomes (Singer and Gottschling 1994). Because this situation is probably incompatible with survival, cells with an indeterminate life span must adopt strategies for telomere conservation. As predicted, cells that emerge from extended life span as immortal cell lines often activate the telomerase enzyme (Counter et al. 1992; Kim et al. 1994).

Cells that are programmed for continuous proliferation generally maintain telomere length. For example,

many stem cell populations possess telomerase activity (Counter et al. 1992; Kim et al. 1994). Telomerase is also induced in mitogen-stimulated lymphocytes and is detected in mitotically active regions of hair follicles and intestinal crypts (for review, see Greider 1998). The association of telomerase with cell proliferation has led to the hypothesis that telomere maintenance is simply a housekeeping function. However, proliferating normal cells in culture generally lack telomerase activity (Counter et al. 1992; Kim et al. 1994).

Stabilization of telomeric repeats may be a prerequisite for tumorigenesis (Counter et al. 1992). Consistent with this notion, telomerase is activated in a high percentage of late-stage human tumors and is present in most tumor-derived cell lines in culture (Counter et al. 1992, 1994; Kim et al. 1994; Shay and Wright 1996). To test the role of telomere maintenance in tumorigenesis, we surveyed known oncogenes for their ability to activate telomerase.

**Results and Discussion***Myc activates telomerase*

Normal human mammary epithelial cells (HMECs) lack telomerase, whereas immortal HMEC-derivatives and breast tumor cell lines are almost universally telomerase-positive (Shay et al. 1995; Bryan and Reddel 1997; Shay and Bacchetti 1997). Introduction of HPV-16 E6 protein into primary HMECs stimulates telomerase activity, suggesting that, in these cells, a single genetic event can potentiate the enzyme (Shay et al. 1993; Klingelutz et al. 1996; Fig. 1A,C and 2). Therefore, we asked whether increased expression of other cellular or viral oncogenes could induce telomerase in HMECs. Ectopic expression of *mdm-2* failed to induce telomerase, consistent with the observation that activation of telomerase by E6 is separable from the ability of E6 to promote the degradation of p53 (Klingelutz et al. 1996; Fig. 2). Several other cellular and viral oncoproteins, including E7, activated Ras (V12), cyclin D1, cdc25C, and cdc25A, also failed to induce telomerase (Fig. 2). However, introduction of a c-Myc expression cassette stimulated telomerase activity in HMECs (Figs. 1A and 2). Enzyme activity was elevated within one passage after transduction of HMECs with a retrovirus that directs Myc expression (Fig. 1C). The Myc-expressing populations displayed levels of telomerase activity that approximated those seen in breast carcinoma cell lines (Fig. 1A; e.g., T47D).

Introduction of E6 into normal human diploid fibroblasts failed to activate telomerase (Klingelutz et al. 1996; Shay et al. 1993; Figs. 1B,C and 2). Similar results were observed for E1A (Fig. 2), activated Ras (V12, not shown), or a dominant-negative p53 allele (Fig. 2). However, telomerase was induced by transduction of either IMR-90 (Figs. 1B,C and 2) or WI-38 cells (Fig. 1C) with a retrovirus that directs c-Myc expression. As with HMECs, activity was apparent immediately after drug

[Key Words: Myc; telomerase; hEST2; tumorigenesis]

<sup>3</sup>Corresponding author.

E-MAIL hannon@cshl.org; FAX (516) 367-8874.

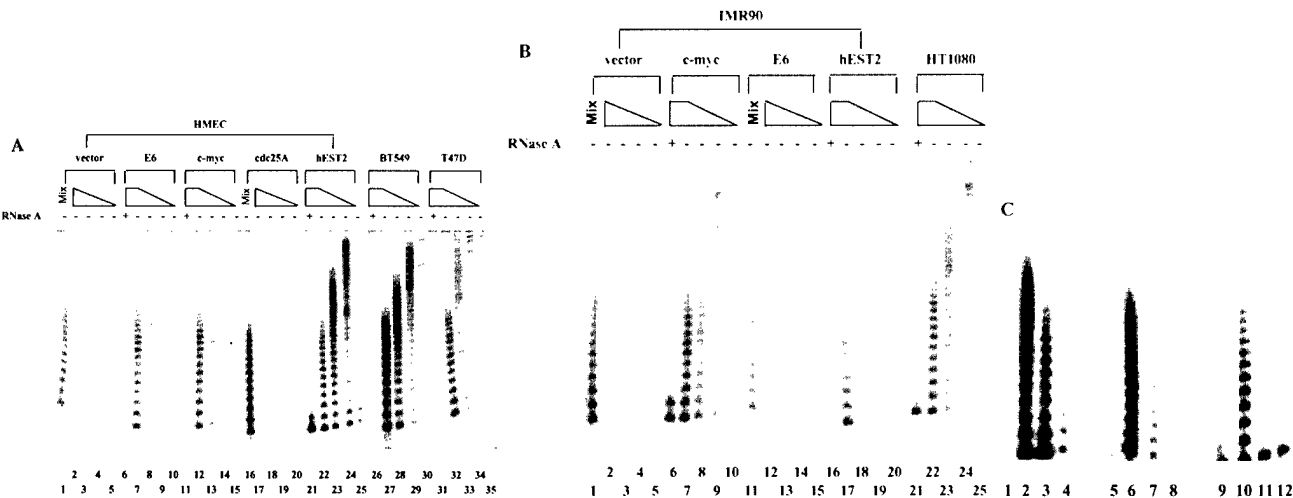


**Figure 1.** Myc activates telomerase. (A) Primary HMECs at passage 12 were infected with empty vector (lanes 1–5), E6 (lanes 6–10), c-Myc (lanes 11–15), cdc25A (lanes 16–20), or hEST2 (lanes 21–25) viruses. Breast cancer cell lines BT549 (lanes 26–30) and T47D (lanes 31–35) were included for comparison. TRAP assays contained lysates from 10,000 (lanes 2,6,7,11,12,17,21,22,26,27,31,32), 1000 (lanes 3,8,13,18,23,28,33), 100 (lanes 4,9,14,19,24,29,34), or 10 (lanes 5,10,15,20,25,30,35) cells. (– and +) Absence or presence of RNase A, respectively. (Mix; lanes 1,16) To exclude the presence of inhibitors in apparently negative lysates, lysate from 10,000 of the indicated cells was mixed with lysate from 10,000 c-Myc-expressing cells. (B) IMR90 cells at passage 14 were infected with empty vector (lanes 1–5), c-Myc (lanes 6–10), E6 (lanes 11–15), or hEST2 (lanes 16–20) viruses. HT1080 cells (lanes 21–25) were included for comparison. TRAP assays were performed with decreasing cell equivalents as in A. (C) HMEC (lanes 1–4), IMR90 (lanes 5–8), or WI38 (lanes 9–12) cells were infected with empty vector (lanes 1,5,9), hEST2 (lanes 2,6,10), c-Myc (lanes 3,7,11), or E6 viruses (lanes 4,8,12). Cells were selected for ~5 days with puromycin or hygromycin and then lysed for telomerase assay. Each lane corresponds to 10,000 cells.

selection (Fig. 1C). The Myc-expressing cells contained levels of telomerase activity comparable to those seen in a telomerase-positive fibrosarcoma cell line, HT1080 (Fig. 1B).

Although c-Myc expression elevates telomerase in both normal epithelial cells and in normal fibroblasts, the HPV-16 E6 protein has been shown to affect telomerase only in epithelial cells (Klingelhutz et al. 1996). Therefore, we questioned the basis of cell-type specific telomerase activation by E6. A recent report suggesting that E6 can activate the Myc promoter (Kinoshita et al. 1997) prompted us to ask whether E6 might regulate telomerase through an effect on Myc expression. In

HMECs, expression of E6 induced Myc to levels approaching those achieved upon transduction of HMECs with a Myc retrovirus (Fig. 3A). Surprisingly, E6-induced alterations in Myc protein did not reflect changes in the abundance of *myc* mRNA (Fig. 3B). Therefore, Myc expression must be controlled post-transcriptionally by E6 in HMECs. In contrast, Myc levels remained unaltered following expression of E6 in IMR-90 cells wherein E6 is incapable of activating telomerase (Fig. 3A). Although E6

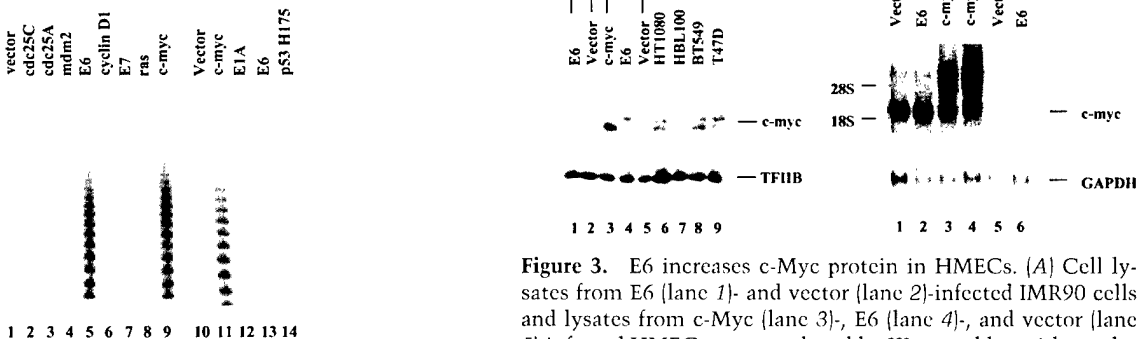


**Figure 2.** Oncogene activation of telomerase. HMECs (lanes 1–9) or IMR90 cells (lanes 10–14) were infected with viruses that direct the expression of the indicated oncogenes (lanes 2–9,11–14) or empty vector (lanes 1,10). Cell extracts were analyzed by TRAP assay.

**Figure 3.** E6 increases c-Myc protein in HMECs. (A) Cell lysates from E6 (lane 1)- and vector (lane 2)-infected IMR90 cells and lysates from c-Myc (lane 3)-, E6 (lane 4)-, and vector (lane 5)-infected HMECs were analyzed by Western blot with a polyclonal Myc antibody. Tumor cell lines, HT1080 (lane 6), HBL100 (lane 7), BT549 (lane 8), and T47D (lane 9), were included for comparison. The expression of TFIIIB was used to normalize loading. (B) Northern analysis of Myc RNA levels in total RNA. GAPDH was probed as a loading control.

may regulate telomerase by other mechanisms, this result is consistent with a model in which E6 regulates telomerase in HMECs by altering the abundance of Myc.

#### *Myc induces hEST2*

The presence of the mRNA encoding hEST2, the catalytic subunit of telomerase, strictly correlates with telomerase activity. The hEST2 message is undetectable in normal tissue and in normal cell lines but is present in immortal and tumor-derived cell lines [Harrington et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997]. Moreover, hEST2 expression and telomerase are suppressed concomitantly when cells are induced to differentiate [Meyerson et al. 1997]. These results suggest that

availability of hEST2 may limit telomerase activity, as was demonstrated recently in a number of normal cell lines [Weinrich et al. 1997; Bodnar et al. 1998]. Expression of hEST2 could also induce telomerase in HMECs and WI38 and IMR-90 cells [Fig. 1A–C]. Activity was apparent immediately following selection of hEST2-expressing cells [Fig. 1C], and the level of telomerase activity observed in hEST2-expressing populations consistently exceeded that observed in cell populations containing c-Myc [Fig. 1A–C].

Because increased expression of hEST2 was sufficient to activate telomerase in both HMECs and in IMR-90 cells [Fig. 1], we asked whether Myc activates telomerase through an effect on hEST2. As expected, hEST2 mRNA was not detectable in normal HMECs, but was induced

at least 50-fold following transduction with a Myc retrovirus [Fig. 4A]. Thus, Myc regulates telomerase by controlling the expression of a limiting telomerase subunit. Because Myc enhances the expression of responsive genes, its action on hEST2 could be either direct or indirect.

#### *hEST2 increases replicative life span in HMECs but not in IMR-90 cells*

Preservation of telomeric repeats requires either the telomerase enzyme or the activation of an alternative pathway for telomere maintenance [Kim et al. 1994; Broccoli et al. 1995; Strahl and Blackburn 1996; Wright et al. 1996; Bryan and Reddel 1997]. In addition, telomere length can be controlled by telomere-binding proteins [van Steensel and de Lange 1997]. To determine whether activation of telomerase in HMECs is sufficient to stabilize telomere length, we followed telomeric restriction fragment (TRF) size as HMECs proliferated either in the presence or absence of telomerase activity. In normal HMECs, telomere length and the abundance of telomeric sequences diminished slightly as cells underwent multiple rounds of division [Fig. 4B]. Activation of telomerase by expression of hEST2 not only prevented telomere shrinkage but also increased both the overall abundance of telomeric sequences and the average length of telomeres [Fig. 4B]. In Myc-expressing cells, however, the abundance of telomeric sequences was intermediate between that observed in cells expressing hEST2 and that observed in control cells [Fig. 4C]. Telomere lengths followed a similar pattern [Fig. 4C].

Generally, in tumors and in immortal cell lines, telomeres are short but stable [Hastie et al. 1990]. Comparison of TRF levels in Myc-expressing HMECs to those in early-passage HMECs suggested that Myc probably stabilized telomeres rather than promoted an increase in TRFs as occurs in hEST2-expressing

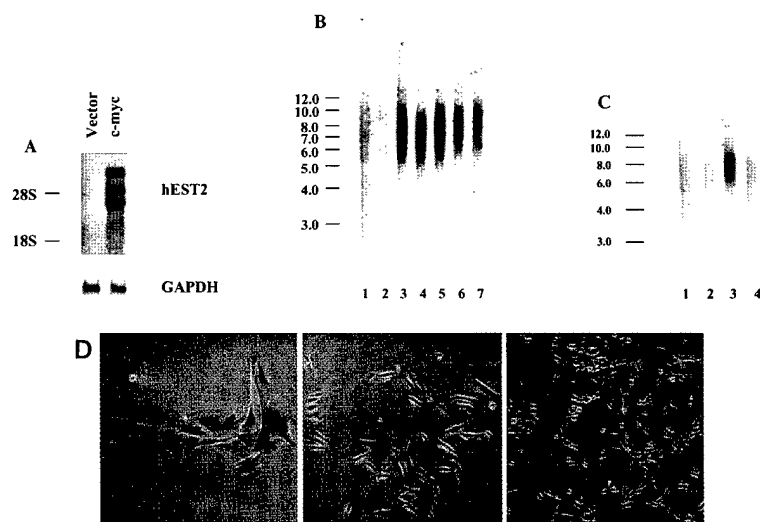


**Figure 4.** Myc regulates hEST2 and extends cellular life span in HMECs. (A) hEST2 Northern analysis of poly(A)<sup>+</sup> RNA from normal HMECs and from HMECs that had been infected with a Myc retrovirus. A Northern blot with GAPDH was performed as a loading control. (B) Genomic DNA (3  $\mu$ g) from early-passage HMECs (passage 12, lane 1), late-passage HMECs (passage 26, lane 2), and hEST2-expressing HMECs [infected at passage 12 and cultured for 3 [lane 3], 6 [lane 4], 8 [lane 5], 10 [lane 6], or 14 [lane 7] additional passages] was digested with *Rsa*I and *Hinf*I. Fragments were separated on a 0.8% agarose gel, and telomeric restriction fragments were visualized with a <sup>32</sup>P-labeled human telomeric sequence (TTAGGG)<sub>3</sub> probe. (C) Genomic DNA (3  $\mu$ g) from early-passage HMECs (passage 12, lane 1), vector-infected HMEC (infected at passage 12 and cultured for six additional passages or ~12–14 PD, lane 2), hEST2-expressing HMECs (infected at passage 12 and cultured for six additional passages or ~12–14 PD, lane 3), and Myc-expressing cells (infected at passage 12 and cultured for six additional passages or ~18 PD, lane 4) were digested with *Rsa*I and *Hinf*I. Fragments were probed with a telomeric probe as described in B. TRF intensity was quantitated on a Fuji BAS2000 PhosphorImager. Normalizing vector-containing HMECs (lane 2) to 100 units of intensity, both early passage HMECs (lane 1) and Myc-expressing HMECs (lane 4) gave ~150 units of intensity and hEST2-expressing HMECs (lane 3) gave ~200 units of intensity. (D) HMECs transduced with empty vector (left), hEST2 (middle), or c-Myc viruses (right) were grown to a PDL of ~56–60. At this PDL, vector cells adopted a senescent morphology and ceased growth. Cells expressing c-Myc and hEST2 continued to proliferate. To assess the percentage of senescent cells in the population, each culture was stained for senescence-associated  $\beta$ -galactosidase. Greater than 95% of the vector-containing cells were  $\beta$ -galactosidase positive whereas <10% of cells expressing hEST2 or Myc were stained.

HMECs (Fig. 4C). Thus, alterations in telomere dynamics after Myc transduction mimic the situation in tumors. Telomerase was ~10-fold more active in hEST2-expressing cells than in Myc-expressing cells. Thus, differences in telomerase activity likely reflect differences in hEST2 expression as the abundance of viral hEST2 mRNA greatly exceeded native hEST2 mRNA levels present in either Myc-expressing HMECs or in any of the tumor cell lines tested to date.

Telomere length has been proposed as the counting mechanism that determines the replicative life span of a cell (Harley 1990; Harley et al. 1991). At a population doubling level (PDL) of ~55–60, vector-containing HMECs ceased proliferation, adopted a senescent morphology (for review, see Stein and Dulic 1995), and stained positive for senescence-associated  $\beta$ -galactosidase (Dimri et al. 1995; Fig. 4D). These cells also showed increased expression of PAI, another senescence marker (Goldstein et al. 1994; data not shown). In contrast, normal HMECs that had received either hEST2 or c-Myc at early passage displayed an extended life span. Cells expressing either c-Myc or hEST2 continued to proliferate beyond the normal senescence point and did not show evident  $\beta$ -galactosidase staining or increased PAI expression (Fig. 4D; data not shown). In both c-Myc and hEST2-expressing cell populations, <10% of cells showed any senescence-associated phenotype at the point at which vector-infected cells senesced. Furthermore, neither population has shown any accumulation of senescent cells during subsequent growth. At present, hEST2- and Myc-expressing populations are a minimum of 40 population doublings (PD) beyond the normal senescence point.

In IMR-90 cells, the consequences of telomerase activation differed from those observed in HMECs. Although hEST2 induced telomerase activity to high levels in early-passage (p14) IMR-90 cells (Fig. 1B,C), this activity was not accompanied by an increase in either the abundance or the length of telomeric sequences (Fig. 5A). To examine the consequences of telomerase activation in normal fibroblasts, vector-containing IMR-90 cells and telomerase-positive hEST2-expressing IMR-90 cells were cultured continuously until the replicative life span of the normal IMR-90 cells was exhausted. Consistent with the idea that telomeres but not telomerase activity per se regulate replicative senescence, hEST2 expression failed to alter the life span of IMR-90 cells. These cells entered replicative senescence within two passages of the point at which normal IMR-90 cells senesced (Fig. 5B). Even after >1 month of maintenance, not a single cell from a population of  $>10^6$  cells escaped the senescence block. Senescence was not attributable to a loss of telomerase as the arrested hEST2-expressing IMR-90 population maintained activity (Fig. 5C).

In contrast, IMR-90 cells engineered to express c-Myc display an extended life span (Fig. 5B), even though these

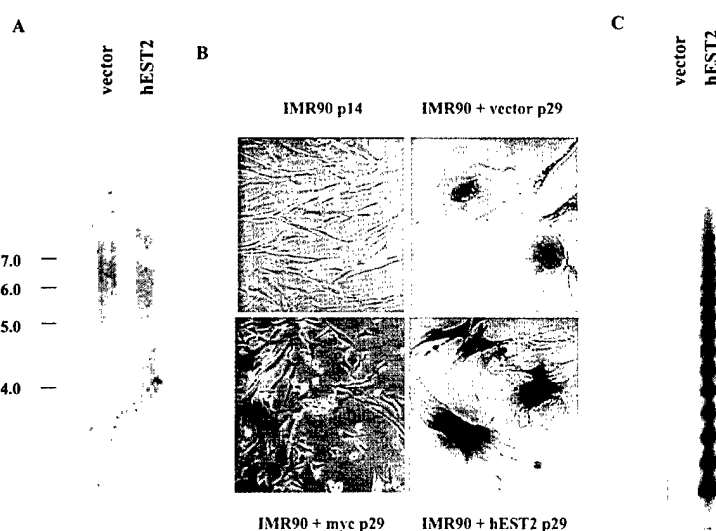


**Figure 5.** Telomerase activation does not affect life span in IMR-90 cells. (A) TRF length of senescent vector-containing IMR-90 and hEST2-expressing IMR-90 cells was analyzed as in Fig. 4. (B) Early-passage IMR-90 cells (passage 14) were infected with empty vector, a hEST2 retrovirus, or a Myc retrovirus as indicated. Cells were passaged until the vector-infected cells reached senescence (~15 additional passages). At this time, hEST2 cells also senesced, but Myc-expressing IMR-90 cells continued to proliferate. Shown are senescence-associated  $\beta$ -galactosidase stains of early-passage IMR-90 cells, senescent vector-containing IMR-90 cells, senescent hEST2-expressing IMR-90 cells and Myc-expressing IMR-90 cells that have bypassed the senescence point and entered extended life span. (C) Telomerase assays of lysates derived from senescent vector-containing IMR-90 and hEST2-expressing IMR-90 populations. Each lane corresponds to 10,000 cells.

cells do not show an obvious stabilization of telomeres. At present Myc-expressing IMR-90 populations have grown for >17 passages (~68 PD) beyond the normal senescence point. Therefore, Myc can extend the life span of a cell even when telomerase activation fails to do so.

These results indicate that the ability of telomerase to extend life span is not universal. Telomerase-positive cells may senesce and still maintain telomerase activity. The mechanisms that regulate the ability of telomerase to extend telomeres and life span may provide an additional level of control over indefinite proliferation and thus tumorigenesis. Furthermore, telomerase-negative cells may adopt alternative strategies for telomere maintenance [alternative lengthening of telomeres (ALT); Bryan and Reddel 1997] and, therefore, achieve immortality without activation of telomerase. The long-term strategy for telomere maintenance adopted by an individual cell will likely depend on the constellation of genetic alterations that such a cell acquires along the pathway to immortality and possibly neoplastic transformation.

The *myc* oncogene is activated by overexpression, gene amplification, translocation, and possibly mutation in a wide variety of different tumor types (Alitalo et al. 1987). Because Myc can elevate telomerase in normal epithelial and fibroblast cells to a level approximating that observed in tumor cell lines, increased Myc activity could account for the presence of telomerase in many

late-stage tumors. In this regard, a study of 100 neuroblastomas revealed that ~20% (16/100) had exceptionally high telomerase activity. Of these, 11 showed amplification of the N-Myc locus (Hiyama et al. 1995). Thus, in this case, telomerase levels correlated well with Myc activation. Although the *myc* oncogene may induce telomerase in a significant proportion of tumors, telomerase may also be regulated by other pathways that contribute to transformation (Holt et al. 1997).

Although telomerase activation has been suggested to be a housekeeping component of a variety of proliferative programs [Greider 1998], oncogenic transformation is often achieved through constitutive activation of elements of normal growth control. In this regard, Myc expression accompanies the proliferation of diverse cell types *in vivo*, and there is significant overlap between contexts in which Myc is expressed and contexts in which telomerase is detected in normal cells. For example, mitogenic stimulation of normal lymphocytes increases Myc levels [Lacy et al. 1986; Kelly and Siebenlist 1988], and stimulated lymphocytes express telomerase (for review, see Greider 1998). Telomerase activity and Myc are also found in human endometrial tissues during the menstrual cycle. Coincidentally, both Myc and telomerase are high during the proliferative phase but are low during the secretory phase [Odom et al. 1989; Kyo et al. 1997]. Conversely, Myc is lost as proliferating cells differentiate and exit the cell cycle (e.g., HL-60; Mitchell et al. 1992). Differentiation of these same cells results in loss of both hEST2 expression and telomerase [Meyerson et al. 1997].

The results presented here, considered together with the overlap between Myc activation and telomerase expression in normal tissues, suggest a model in which telomerase may respond to Myc both during the execution of normal proliferation programs and in tumors. Promotion of cell proliferation and oncogenic transformation by Myc probably requires induction of a number of different target genes (for review, see Grandori and Eisenman 1997). In fact, we show that Myc can bypass replicative senescence under circumstances in which telomerase activation alone is ineffective. Thus, telomerase activity in tumors may simply reflect activation of oncogenes such as Myc. However, it is likely that telomere maintenance contributes to the long-term proliferative potential of tumor cells, and therefore telomerase activation may be one component of the ability of Myc to facilitate tumor formation.

## Materials and methods

### Retroviral plasmids

The following viral plasmids were used: pBabe-puro (Morgenstern and Land 1990), MarXII-hygro, mouse c-myc/MarXII-hygro, mdm-2/MarXII-hygro (from Dr. P. Sun, CSHL), E6/pBabe-puro, cdc25A/MarXII-hygro, cyclin D1/pBabe-puro, rasV12/pBabe-puro, E1A/pWz1-hygro, p53175H/pWz1-hygro, cdc25C/pBabe-puro, and E7/pBabe-puro. The full-length hEST2 cDNA (from Dr. R. Weinberg, MIT, Cambridge, MA) was cloned into pBabe-puro vector at the *EcoRI* and *SalI* sites.

### Cell culture and retroviral-mediated gene transfer

HMEC 184 spiral K cells were from Dr. M. Stampfer (Lawrence Berkeley Laboratory, Berkeley, CA); IMR90 and WI38 and human breast cancer

cell lines BT549, T47D, and HBL100 were from ATCC; and HT1080 cells were from G. Stark (Cleveland Clinic Foundation, OH). The amphotropic packaging line, LinX-A, was produced in our laboratory (L.Y. Xie, D. Beach, and G. Hannon, unpubl.). HMEC were cultured in complete mammary epithelium growth medium [MEGM] (Clonetics). Fibroblasts and LinX-A cells were maintained in DMEM (GIBCO-BRL) plus 10% FBS (Sigma). BT549, HBL100, and T47D were maintained as directed by the supplier. LinX-A cells were transfected by calcium-phosphate precipitation with a mixture containing 15  $\mu$ g of retroviral plasmid and 15  $\mu$ g of sonicated salmon sperm DNA. Transfected cells were incubated at 37°C for 24 hr and then shifted to 30°C for virus production. After 48 hr, virus was collected, and the virus-containing medium was filtered to remove packaging cells (0.45- $\mu$ m filter; Millipore). Target cells were infected with virus supernatants supplemented with 4  $\mu$ g/ml polybrene (Sigma) by centrifugation for 1 hr at 1000g and incubation at 30°C overnight. Infected cells were selected 48 hr after infection with the appropriate drugs (hygromycin, G418, or puromycin).

### Telomerase assays and expression analyses

[illegible]

TRF length was measured as described by Strahl and Blackburn (1996) and senescence-associated  $\beta$ -galactosidase activity was determined as described by Serrano et al. (1997).

For Northern blotting, total RNA was isolated from subconfluent cultures by use of Trizol reagent (GIBCO-BRL). Total RNA (10 µg) or poly(A)<sup>+</sup> RNA (5 µg) was resolved by electrophoresis and transferred to Hybond-N<sup>+</sup> membranes according to the manufacturer's instructions. hEST2 was visualized after hybridization with a labeled *Stu*I fragment of hEST2 (Meyerson et al. 1997). Myc and GAPDH were visualized with probes derived from cDNAs.

Western blotting was performed essentially as described by Harlow and Lane (1988). Cells were washed with cold PBS and lysed in Laemmli loading buffer. Lysates were heated at 95°C for 10 min. Samples were separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were incubated with either a c-Myc rabbit polyclonal antibody [N-262; Santa Cruz] or a TFIIB rabbit polyclonal antibody (from Dr. B. Tansey, CSHL). Immune complexes were visualized by secondary incubation with <sup>125</sup>I-labeled protein A (ICN).

## Acknowledgments

We thank R. Weinberg for providing the full-length hEST2 cDNA. HMECs were a kind gift of M. Stamper. We thank B. Tansey for helpful discussions and for providing the TFIIB antibody. This work was supported by a grant from the U.S. Army Breast Cancer research program (DAMD17-96-1-6053) and in part by funds from the National Institutes of Health. D. Beach is supported by the Hugh and Catherine Stevenson Fund. G. Hannon is a Pew Scholar in the Biomedical Sciences.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

## References

- Alitalo, K., P. Koskinen, T.P. Makela, K. Saksela, L. Sistonen, and R. Winqvist. 1987. myc oncogenes: Activation and amplification. *Biochim. Biophys. Acta* **907**: 1-32.
- Bodnar, A.G., M. Ouellette, M. Frolkis, S.E. Holt, C.P. Chiu, G.B. Morin, C.B. Harley, J.W. Shay, S. Lichtsteiner, and W.E. Wright. 1998. Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**: 349-352.
- Broccoli, D., J.W. Young, and T. de Lange. 1995. Telomerase activity in

- normal and malignant hematopoietic cells. *Proc. Natl. Acad. Sci.* **92**: 9082-9086.
- Bryan, T.M. and R.R. Reddel. 1997. Telomere dynamics and telomerase activity in in vitro immortalised human cells. *Eur. J. Cancer* **33**: 767-773.
- Counter, C.M., A.A. Avilion, C.E. LeFeuvre, N.G. Stewart, C.W. Greider, C.B. Harley, and S. Bacchetti. 1992. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.* **11**: 1921-1929.
- Counter, C.M., H.W. Hirte, S. Bacchetti, and C.B. Harley. 1994. Telomerase activity in human ovarian carcinoma. *Proc. Natl. Acad. Sci.* **91**: 2900-2904.
- Dimri, G.P., X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E.E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith et al. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci.* **92**: 9363-9367.
- Goldstein, S., E.J. Moerman, S. Fujii, and B.E. Sobel. 1994. Overexpression of plasminogen activator inhibitor type-1 in senescent fibroblasts from normal subjects and those with Werner syndrome. *J. Cell. Physiol.* **161**: 571-579.
- Grandori, C. and R.N. Eisenman. 1997. Myc target genes. *Trends Biochem. Sci.* **22**: 177-181.
- Greider, C.W. 1998. Telomerase activity, cell proliferation, and cancer. *Proc. Natl. Acad. Sci.* **95**: 90-92.
- Harley, C.B. 1991. Telomere loss: Mitotic clock or genetic time bomb? *Mutat. Res.* **256**: 271-282.
- Harley, C.B., A.B. Futcher, and C.W. Greider. 1990. Telomeres shorten during aging of human fibroblasts. *Nature* **345**: 458-460.
- Harlow, E.L. and D. Lane. 1988. *Antibodies: A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Harrington, L., W. Zhou, T. McPhail, R. Oulton, D.S. Yeung, V. Mar, M.B. Bass, and M.O. Robinson. 1997. Human telomerase contains evolutionarily conserved catalytic and structural subunits. *Genes & Dev.* **11**: 3109-3115.
- Hastic, N.D., M. Dempster, M.G. Dunlop, A.M. Thompson, D.K. Green, and R.C. Allshire. 1990. Telomere reduction in human colorectal carcinoma and with aging. *Nature* **346**: 866-868.
- Hiyama, E., K. Hiyama, T. Yokoyama, Y. Matsuura, M.A. Piatyszek, and J.W. Shay. 1995. Correlating telomerase activity levels with human neuroblastoma outcomes. *Nature Med.* **1**: 249-255.
- Holt, S.E., W.E. Wright, and J.W. Shay. 1997. Multiple pathways for the regulation of telomerase activity. *Eur. J. Cancer* **33**: 761-766.
- Kelly, K. and U. Siebenlist. 1988. Mitogenic activation of normal T cells leads to increased initiation of transcription in the c-myc locus. *J. Biol. Chem.* **263**: 4828-4831.
- Kim, N.W., M.A. Piatyszek, K.R. Prowse, C.B. Harley, M.D. West, P.L. Ho, G.M. Coviello, W.E. Wright, S.L. Weinrich, and J.W. Shay. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**: 2011-2015.
- Kinoshita, T., H. Shirasawa, Y. Shino, H. Moriya, L. Desbarats, M. Eilers, and B. Simizu. 1997. Transactivation of prothymosin alpha and c-myc promoters by human papillomavirus type 16 E6 protein. *Virology* **232**: 53-61.
- Klingelutz, A.J., S.A. Foster, and J.K. McDougall. 1996. Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature* **380**: 79-82.
- Kyo, S., M. Takakura, T. Kohama, and M. Inoue. 1997. Telomerase activity in human endometrium. *Cancer Res.* **57**: 610-614.
- Lacy, J., S.N. Sarkar, and W.C. Summers. 1986. Induction of c-myc expression in human B lymphocytes by B-cell growth factor and anti-immunoglobulin. *Proc. Natl. Acad. Sci.* **83**: 1458-1462.
- Meyerson, M., C.M. Counter, E.N. Eaton, L.W. Ellisen, P. Steiner, S.D. Caddle, L. Ziaugra, R.L. Beijersbergen, M.J. Davidoff, Q. Liu, S. Bacchetti, D.A. Haber, and R.A. Weinberg. 1997. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell* **90**: 785-795.
- Mitchell, L.S., R.A. Neill, and G.D. Birnie. 1992. Temporal relationships between induced changes in c-myc mRNA abundance, proliferation, and differentiation in HL60 cells. *Differentiation* **49**: 119-125.
- Morgenstern, J.P. and H. Land. 1990. Advanced mammalian gene transfer: High titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* **18**: 3587-3596.
- Nakamura, T.M., G.B. Morin, K.B. Chapman, S.L. Weinrich, W.H. Andrews, J. Lingner, C.B. Harley, and T.R. Cech. 1997. Telomerase catalytic subunit homologs from fission yeast and human. *Science* **277**: 955-959.
- Odom, L.D., J.M. Barrett, C.G. Pantazis, L.D. Stoddard, and P.G. McDonough. 1989. Immunocytochemical study of ras and myc proto-oncogene polypeptide expression in the human menstrual cycle. *Am. J. Obstet. Gynecol.* **161**: 1663-1668.
- Olovnikov, A.M. 1973. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *J. Theor. Biol.* **41**: 181-190.
- Serrano, M., A.W. Lin, M.E. McCurrach, D. Beach, and S.W. Lowe. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**: 593-602.
- Shay, J.W. and S. Bacchetti. 1997. A survey of telomerase activity in human cancer. *Eur. J. Cancer* **33**: 787-791.
- Shay, J.W. and W.E. Wright. 1996. Telomerase activity in human cancer. *Curr. Opin. Oncol.* **8**: 66-71.
- Shay, J.W., W.E. Wright, D. Brasiskyte, and B.A. Van der Haegen. 1993. E6 of human papillomavirus type 16 can overcome the M1 stage of immortalization in human mammary epithelial cells but not in human fibroblasts. *Oncogene* **8**: 1407-1413.
- Shay, J.W., G. Tomlinson, M.A. Piatyszek, and L.S. Gollahon. 1995. Spontaneous in vitro immortalization of breast epithelial cells from a patient with Li-Fraumeni syndrome. *Mol. Cell. Biol.* **15**: 425-432.
- Singer, M.S. and D.E. Gottschling. 1994. TLC1: Template RNA component of *Saccharomyces cerevisiae* telomerase. *Science* **266**: 404-409.
- Stein, G.H. and V. Dulic. 1995. Origins of G1 arrest in senescent human fibroblasts. *BioEssays* **17**: 537-543.
- Strahl, C. and E.H. Blackburn. 1996. Effects of reverse transcriptase inhibitors on telomere length and telomerase activity in two immortalized human cell lines. *Mol. Cell. Biol.* **16**: 53-65.
- van Steensel, B. and T. de Lange. 1997. Control of telomere length by the human telomeric protein TRF1. *Nature* **385**: 740-743.
- Watson, J.D. 1972. Origin of concatemeric T7 DNA. *Nat. New Biol.* **239**: 197-201.
- Weinrich, S.L., R. Pruzan, L. Ma, M. Ouellette, V.M. Tesmer, S.E. Holt, A.G. Bodnar, S. Lichtsteiner, N.W. Kim, J.B. Trager, R.D. Taylor, R. Carlos, W.H. Andrews, W.E. Wright, J.W. Shay, C.B. Harley, and G.B. Morin. 1997. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTERT. *Nature Genet.* **17**: 498-502.
- Wright, W.E., M.A. Piatyszek, W.E. Rainey, W. Byrd, and J.W. Shay. 1996. Telomerase activity in human germline and embryonic tissues and cells. *Dev. Genet.* **18**: 173-179.

# p53-Independent Role of MDM2 in TGF- $\beta$ 1 Resistance

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

Transforming growth factor- $\beta$  (TGF- $\beta$ ) inhibits cell proliferation, and acquisition of TGF- $\beta$  resistance has been linked to tumorigenesis. A genetic screen was performed to identify complementary DNAs that abrogated TGF- $\beta$  sensitivity in mink lung epithelial cells. Ectopic expression of murine double minute 2 rescued TGF- $\beta$ -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- $\beta$  resistance. Thus, MDM2 may confer TGF- $\beta$  resistance in a subset of tumors and may promote tumorigenesis by interference with two independent tumor suppressors, p53 and Rb.

colonies and were morphologically identical to untreated cells (Fig. 1) (9). MDM2 also conferred TGF- $\beta$  resistance in human mammary epithelial cells (HMECs) (Fig. 1, bottom panel).

The isolation of *c-myc*, a gene previously shown to overcome TGF- $\beta$ -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- $\beta$  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- $\beta$  resistance.

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

MDM2 associates with and inactivates the tumor suppressor protein, p53. To test the possibility that MDM2 bypasses TGF- $\beta$ -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

The TGF- $\beta$  signaling pathway has been implicated in tumor suppression (1). Loss of TGF- $\beta$  sensitivity is frequently observed in tumors derived from cells that are normally sensitive, and the extent of TGF- $\beta$  resistance often correlates with malignancy (2). Some tumors may develop TGF- $\beta$  resistance following inactivation of essential components of the TGF- $\beta$  signaling pathway (3-5) or through deletion of the *p15<sup>INK4B</sup>* locus (6).

P. Sun, P. Dong, K. Dai, G. J. Hannon, Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA. D. Beach, the Institute of Child Health, 30 Guilford Street, London WC1N, UK.

\*Present address: Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608, USA.

†To whom correspondence should be addressed. E-mail: d.beach@ich.ucl.ac.uk

However, such alterations cannot account for the majority of cases in which TGF- $\beta$  responsiveness is lost. Therefore, TGF- $\beta$  resistance must also be achieved by other mechanisms.

To identify genetic alterations that lead to TGF- $\beta$  resistance in tumor cells, we screened for genes that, when overexpressed, allow cells to escape TGF- $\beta$ -induced growth arrest (7). A cDNA library was introduced into Mv1Lu, a TGF- $\beta$ -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- $\beta$ . We recovered three genes that conferred TGF- $\beta$  resistance: *Mdm2*, *c-myc*, and *NF-IX-1* (Fig. 1, top panel). When treated with TGF- $\beta$ , cells expressing MDM2, *c-myc*, or NF-IX-1 formed

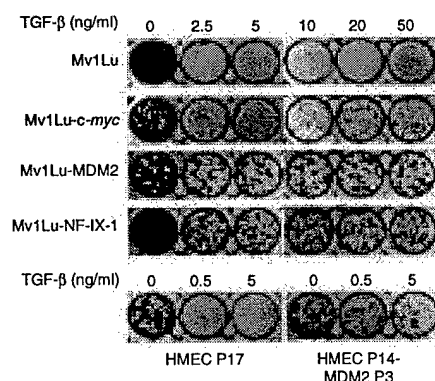


**Fig. 1.** *Mdm2*, *c-myc*, and *NF-IX-1* bypass TGF- $\beta$ -induced growth arrest. (Top panel) Control Mv1Lu or Mv1Lu expressing *c-myc*, MDM2, or NF-IX-1 (4000 cells) were treated with TGF- $\beta$  for 8 days. (Bottom panel) HMECs at passage 14 were infected with a retroviral vector that drives 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- $\beta$  for 16 days. All cells were visualized by staining with crystal violet.

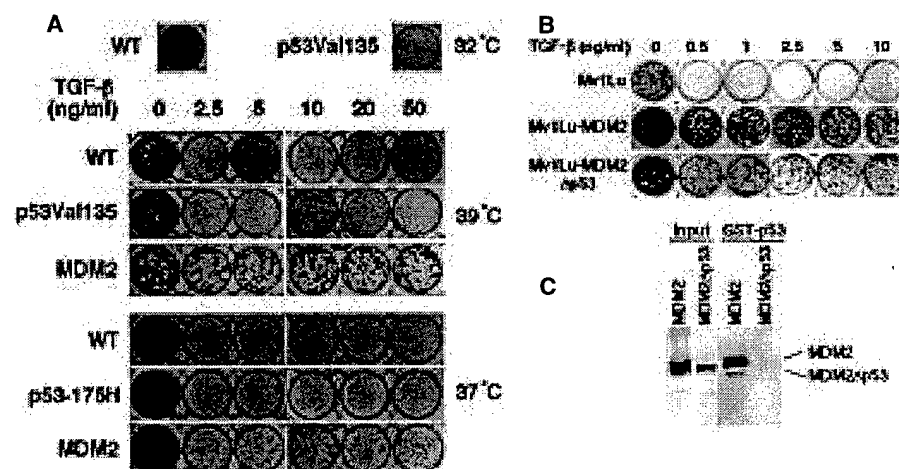


**Fig. 2.** MDM2 confers TGF- $\beta$  resistance through a p53-independent mechanism in Mv1Lu cells. (A) Control Mv1Lu cells or cells expressing MDM2, p53Val135, or p53-175H were treated with TGF- $\beta$  for 8 days. (B) Control Mv1Lu cells or cells expressing MDM2 or an MDM2 mutant that cannot bind p53 were treated with TGF- $\beta$  for 8 days. (C) Wild-type or mutant MDM2 proteins were translated in vitro from pcDNA3 in the presence of [<sup>35</sup>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- $\beta$  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- $\beta$  resistance (Fig. 2B). Thus, MDM2 overcomes TGF- $\beta$  through a mechanism that is distinct from its ability to inactivate p53.

TGF- $\beta$  induces G<sub>1</sub> 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- $\beta$  resistance in Mv1Lu cells (9), we investigated the possibility that MDM2 could bypass TGF- $\beta$  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- $\beta$  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.

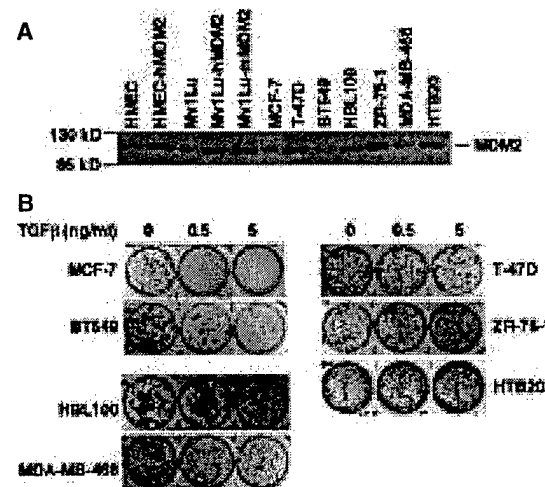
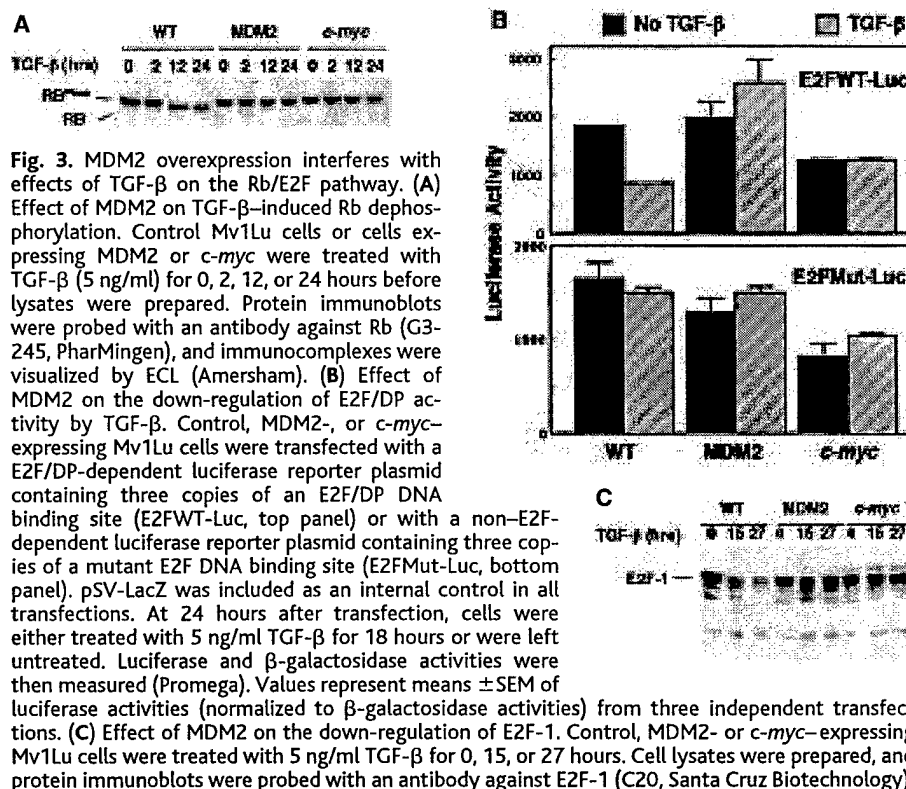
E2F proteins are transcription factors that bind to unphosphorylated Rb. Rb phosphorylation releases E2F proteins in an active, growth-promoting form (23). The effect of MDM2 on Rb phosphorylation predicted that MDM2 would have a positive effect on E2F activity. In contrast to previous studies in other cell lines (21, 22), expression of MDM2 in Mv1Lu cells did not increase the activity of an E2F-dependent reporter construct (Fig. 3B). TGF- $\beta$  treatment reduced transcription of this reporter by twofold. However, MDM2 expression prevented this reduction (Fig. 3B). Alteration of E2F activity by either TGF- $\beta$  treatment or MDM2 overexpression reflected changes in E2F-1 protein levels (Fig. 3C). TGF- $\beta$  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- $\beta$ -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- $\beta$ -induced arrest through overlapping mechanisms.

MDM2 is frequently overexpressed in human tumors (12). We identified one biological consequence of MDM2 overexpression, bypass of TGF- $\beta$ -induced growth arrest. TGF- $\beta$  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- $\beta$  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- $\beta$ -sensitive cells to a resistant phenotype (Fig. 1, bottom panel). These observations raised the possibility that increased MDM2 expression might contribute to TGF- $\beta$  resistance in tumors.

Therefore, we examined the relationship between MDM2 expression levels (Fig. 4A) and TGF- $\beta$  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- $\beta$ -resistant by infection with MDM2 retroviral vectors. These three cell lines were completely resistant to TGF- $\beta$ -induced growth arrest. The two cell lines (MCF-7 and BT549) that were most sensitive to TGF- $\beta$  treatment had very low MDM2 levels, similar to those seen in TGF- $\beta$ -sensitive, normal HMECs. Thus, in several tumor cell lines, increased MDM2 expression strictly correlated with the ability to escape TGF- $\beta$ -induced growth inhibition. Two other breast carcinoma cell lines (HBL100 and MDA-MB-468) exhibited partial resistance to TGF- $\beta$  despite low levels of MDM2 ex-



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

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

# References and Notes

1. Y. Zhu, J. A. Richardson, L. F. Parada, J. M. Graff, *Cell* **94**, 703 (1998).
2. J. Filmus and R. S. Kerbel, *Curr. Opin. Oncol.* **5**, 123 (1993).
3. A. Kimchi, X. F. Wang, R. A. Weinberg, S. Cheifetz, J. Massagué, *Science* **240**, 196 (1988).
4. K. Eppert et al., *Cell* **86**, 543 (1996).
5. M. Schutte et al., *Cancer Res.* **56**, 2527 (1996).
6. S. N. Wagner, C. Wagner, L. Briedigkeit, M. Goos, *Br. J. Dermatol.* **138**, 13 (1998).
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- $\beta$  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-1X*.
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.

9. P. Sun, K. Dai, G. J. Hannon, D. Beach, data not shown.
10. M. G. Alexandrow, M. Kawabata, M. Aakre, H. L. Moses, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3239 (1995).
11. S. Kulkarni and R. M. Gronostajski, *Cell Growth Differ.* **7**, 501 (1996).
12. J. Momand and G. P. Zambetti, *J. Cell. Biochem.* **64**, 343 (1997).
13. D. Michalovitz, O. Halevy, M. Oren, *Cell* **62**, 671 (1990).
14. P. W. Hinds et al., *Cell Growth Differ.* **1**, 571 (1990).
15. M. Hachiya et al., *Anticancer Res.* **14**, 1853 (1994).
16. M. E. Ewen, C. J. Oliver, H. K. Sluss, S. J. Miller, D. S. Peeper, *Genes Dev.* **9**, 204 (1995).
17. M. Laiho, J. A. DeCaprio, J. W. Ludlow, D. M. Livingston, J. Massagué, *Cell* **62**, 175 (1990).
18. J. A. Pietenpol et al., *ibid.* **61**, 777 (1990).
19. J. K. Schwarz et al., *Proc. Natl. Acad. Sci. U.S.A.* **92**, 483 (1995).
20. S. N. Boyer, D. E. Wazer, V. Band, *Cancer Res.* **56**, 4620 (1996).
21. Z. X. Xiao et al., *Nature* **375**, 694 (1995).
22. K. Martin et al., *ibid.* **375**, 691 (1995).
23. P. D. Adams and W. G. Kaelin Jr., *Semin. Cancer Biol.* **6**, 99 (1995).

24. P. C. Nowell and J. S. Moore, *Immunol. Res.* **17**, 171 (1998).
25. P. Schmid, P. Itin, T. Ruffli, *Carcinogenesis* **16**, 1499 (1995).
26. K. Krasagakis, C. Garbe, P. I. Schrier, C. E. Orfanos, *Anticancer Res.* **14**, 2565 (1994).
27. M. Reiss and M. H. Barcellos-Hoff, *Breast Cancer Res. Treat.* **45**, 81 (1997).
28. C. Poremba et al., *Oncol. Res.* **7**, 331 (1995).
29. C. E. Bueso-Ramos et al., *Breast Cancer Res. Treat.* **37**, 179 (1996).
30. M. Jiang et al., *Int. J. Cancer* **74**, 529 (1997).
31. T. Gunther, R. Schneider-Stock, J. Rys, A. Niezabitowski, A. Roessner, *J. Cancer Res. Clin. Oncol.* **123**, 388 (1997).
32. T. Watanabe, A. Ichikawa, H. Saito, T. Hotta, *Leuk. Lymphoma* **21**, 391 (1996).
33. A. Teti et al., *Int. J. Cancer* **72**, 1013 (1997).
34. I. Sigalas, A. H. Calvert, J. J. Anderson, D. E. Neal, J. Lunec, *Nature Med.* **2**, 912 (1996).
35. We thank B. Vogelstein, D. Livingston, A. Levine, and M. Roussel for reagents; Berlex, Inc. for TGF- $\beta$ ; and R. Maestro and D. Conklin for helpful comments. Supported in part by grants from NIH (D.B. and G.J.H.), the Helen Hay Whitney Foundation (P.S.), the U.S. Army (G.J.H.), and the Pew Foundation (G.J.H.). D.B. is supported by the Hugh and Catherine Stevenson Fund.

28 April 1998; accepted 6 November 1998

## Regulation of Cocaine Reward by CREB

William A. Carlezon Jr., Johannes Thome, Valerie G. Olson, Sarah B. Lane-Ladd, Edward S. Brodtkin, Noboru Hiroi, Ronald S. Duman, Rachael L. Neve, Eric J. Nestler\*

Cocaine regulates the transcription factor CREB (adenosine 3',5'-monophosphate response element binding protein) in rat nucleus accumbens, a brain region that is important for addiction. Overexpression of CREB in this region decreases the rewarding effects of cocaine and makes low doses of the drug aversive. Conversely, overexpression of a dominant-negative mutant CREB increases the rewarding effects of cocaine. Altered transcription of dynorphin likely contributes to these effects: Its expression is increased by overexpression of CREB and decreased by overexpression of mutant CREB. Moreover, blockade of  $\kappa$  opioid receptors (on which dynorphin acts) antagonizes the negative effect of CREB on cocaine reward. These results identify an intracellular cascade—culminating in gene expression—through which exposure to cocaine modifies subsequent responsiveness to the drug.

Cocaine causes complex molecular adaptations in brain reward systems, some of which affect its addictive qualities (1). For example, chronic cocaine use increases formation of adenosine 3',5'-monophosphate (cAMP) and activity of cAMP-dependent protein kinase (PKA) in the nucleus accumbens (2), a neural substrate for the rewarding actions of cocaine (3, 4). Stimulation of PKA in the nucleus

accumbens counteracts the rewarding properties of cocaine (5), which suggests a neural mechanism of drug tolerance. Increased PKA activity would be expected to lead to increased phosphorylation of CREB, which mediates many of the effects of cAMP and PKA on gene expression (6, 7). However, direct evidence for a role of CREB in cocaine actions has been lacking. To address this issue, we selectively induced CREB overexpression in the nucleus accumbens with microinjections of a herpes simplex virus vector (HSV-CREB) and measured alterations in the rewarding properties of cocaine with place conditioning (8). We performed the same experiments in other rats after overexpression of a dominant negative mutant CREB (mCREB) (8), which contains a single point

W. A. Carlezon Jr., J. Thome, V. G. Olson, S. B. Lane-Ladd, E. S. Brodtkin, N. Hiroi, R. S. Duman, E. J. Nestler, Division of Molecular Psychiatry, Center for Genes and Behavior, Yale University School of Medicine and Connecticut Mental Health Center, 34 Park Street, New Haven, CT 06508, USA. R. L. Neve, Department of Genetics, Harvard Medical School, McLean Hospital, 115 Mill Street, Belmont, MA 02178, USA.

\*To whom correspondence should be addressed.

Reprint Series  
19 February 1999, Volume 283

**SCIENCE**

# **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

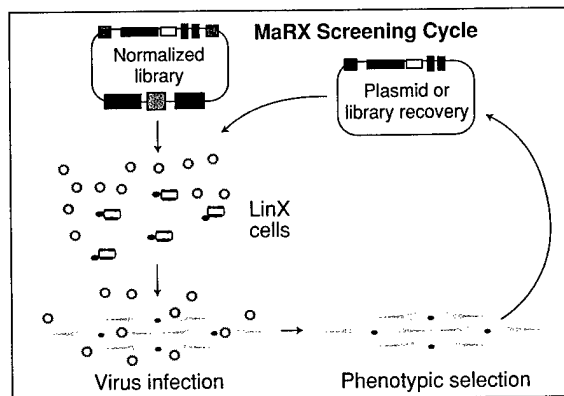


**Fig. 1.** The MaRX cycle. A normalized DNA library is converted into a library of retroviruses by using a packaging cell line (linX). Then, these infected recipient cells are selected or enriched on the basis of a specific biological property. Proviruses are recovered and used for virus production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

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.

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 exist-

ing 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.

G. J. Hannon is at Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, and Genetica Inc., Post Office Box 99, Cold Spring Harbor, NY 11724, USA. P. Sun and D. S. Conklin are at Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA. A. Carnero is at Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA, and The Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK. L. Y. Xie is at Genetica Inc., Post Office Box 99, Cold Spring Harbor, NY 11724, USA. R. Maestro is at Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA, and Centro di Riferimento Oncologico, 33081 Aviano, Italy. D. Beach is at The Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK, and Genetica Inc., Post Office Box 99, Cold Spring Harbor, NY 11724, USA.

\*To whom correspondence should be addressed. E-mail: beach@genetica1.com

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 trans-

ferred 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 anti-

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.

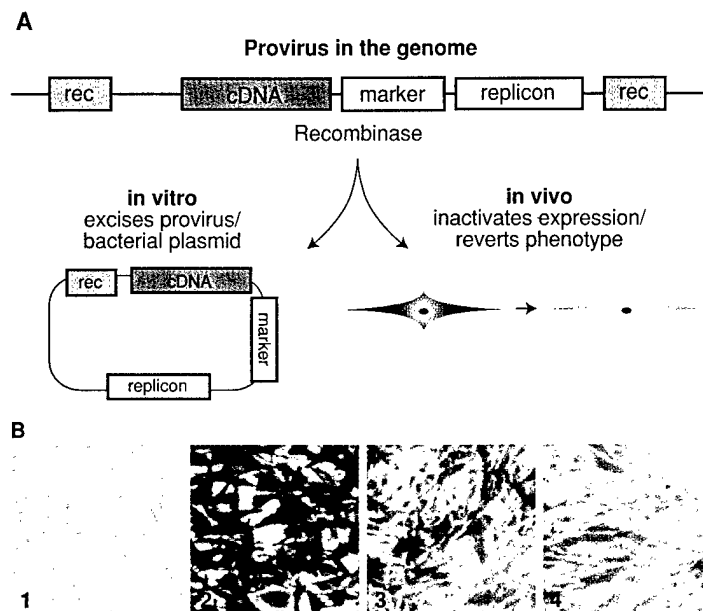


**Fig. 2.** Recombinase excision of the MaRX vector. (A) A representation of the integrated MaRX provirus and the consequences of recombinase treatment in vitro and in vivo. (B) NIH-3T3 cells (1) were infected with a MaRX virus that directs  $\beta$ -galactosidase expression (2). These cells were then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

sense *p53* mini-library allowed colony formation at a frequency of roughly  $10^{-3}$  per cell.

Provirus 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.

## References and Notes

- G. W. Beadle and E. L. Tatum, *Proc. Natl. Acad. Sci. U.S.A.* **27**, 541 (1941).
- J. D. Beggs, *Nature* **275**, 104 (1978).
- A. Hinne, J. B. Hicks, G. R. Fink, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1929 (1978).
- D. Beach and P. Nurse, *Nature* **290**, 140 (1981).
- L. P. Deiss, E. Feinstein, H. Berissi, O. Cohen, A. Kimchi, *Genes Dev.* **9**, 15 (1995).
- I. Whitehead, H. Kirk, R. Kay, *Mol. Cell. Biol.* **15**, 704 (1995).
- J. R. Rayner and T. J. Gonda, *ibid.* **14**, 880 (1994).
- M. Goldfarb, K. Shimizu, M. Peruchio, M. Wigler, *Nature* **296**, 404 (1982).
- M. L. Slater and H. L. Ozer, *Cell* **7**, 289 (1976).
- A. V. Gudkov et al., *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3231 (1993).
- W. Nellen and G. Sczakiel, *Mol. Biotechnol.* **6**, 7 (1996).
- A. Carnero et al., unpublished observations.
- P. Sun et al., *Science* **282**, 2270 (1998).
- R. Maestro et al., unpublished observations.
- J. Hudson et al., unpublished observations.
- D. S. Conklin et al., unpublished observations.
- P. Sun et al., unpublished observations.
- Supported in part by the U.S. Army (G.J.H.), the Pew Foundation for Biomedical Research (G.J.H.), the Helen Hay Whitney Fund (P.S.), the Damon Runyon-Walter Winchell Cancer Research Fund (D.S.C.), the Hugh and Catherine Stevenson Trust (D.B.), NIH (G.J.H., D.B.), and Genetica, Inc.

# *twist* is a potential oncogene that inhibits apoptosis

Roberta Maestro,<sup>1,2</sup> Angelo P. Dei Tos,<sup>3</sup> Yasuo Hamamori,<sup>4</sup> Svetlana Krasnokutsky,<sup>2</sup> Vittorio Sartorelli,<sup>4</sup> Larry Kedes,<sup>4</sup> Claudio Doglioni,<sup>5</sup> David H. Beach,<sup>6</sup> and Gregory J. Hannon<sup>2,7</sup>

<sup>1</sup>Experimental Oncology 1, Centro di Riferimento Oncologico, 33081 Aviano, Italy; <sup>2</sup>Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724 USA; <sup>3</sup>Department of Histopathology, Treviso City Hospital, Treviso, Italy; <sup>4</sup>Institute for Genetic Medicine, University of Southern California School of Medicine, Los Angeles, California 90033 USA; <sup>5</sup>Department of Histopathology, Belluno City Hospital, Belluno, Italy; <sup>6</sup>Institute of Child Health, London WC1N 1EH, UK

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]

Received March 19, 1999; revised version accepted July 7, 1999.

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

<sup>7</sup>Corresponding author.  
E-MAIL hannon@cshl.org; FAX (516) 367-8874.

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 coinfecting 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

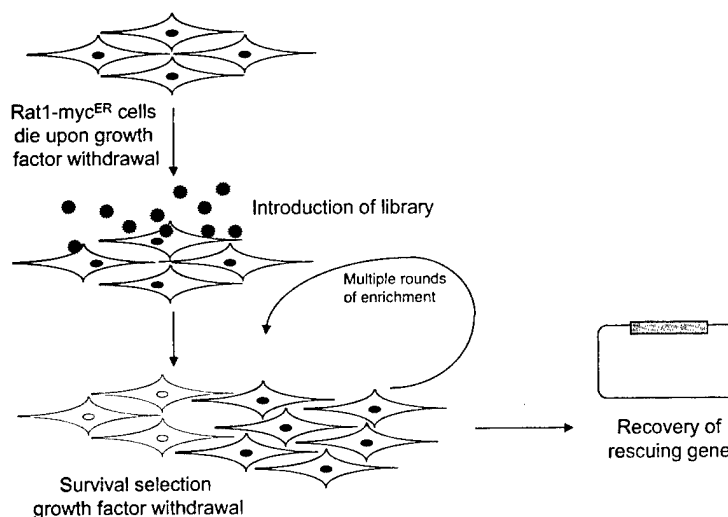


**Figure 1.** Schematic outline of the screen designed to identify genes that confer protection from Myc-induced apoptosis.

conditions. The proportion of LacZ/Bcl-2 cells was monitored following each round, and after four cycles,  $\beta$ -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  $\sim 5 \times 10^6$  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).

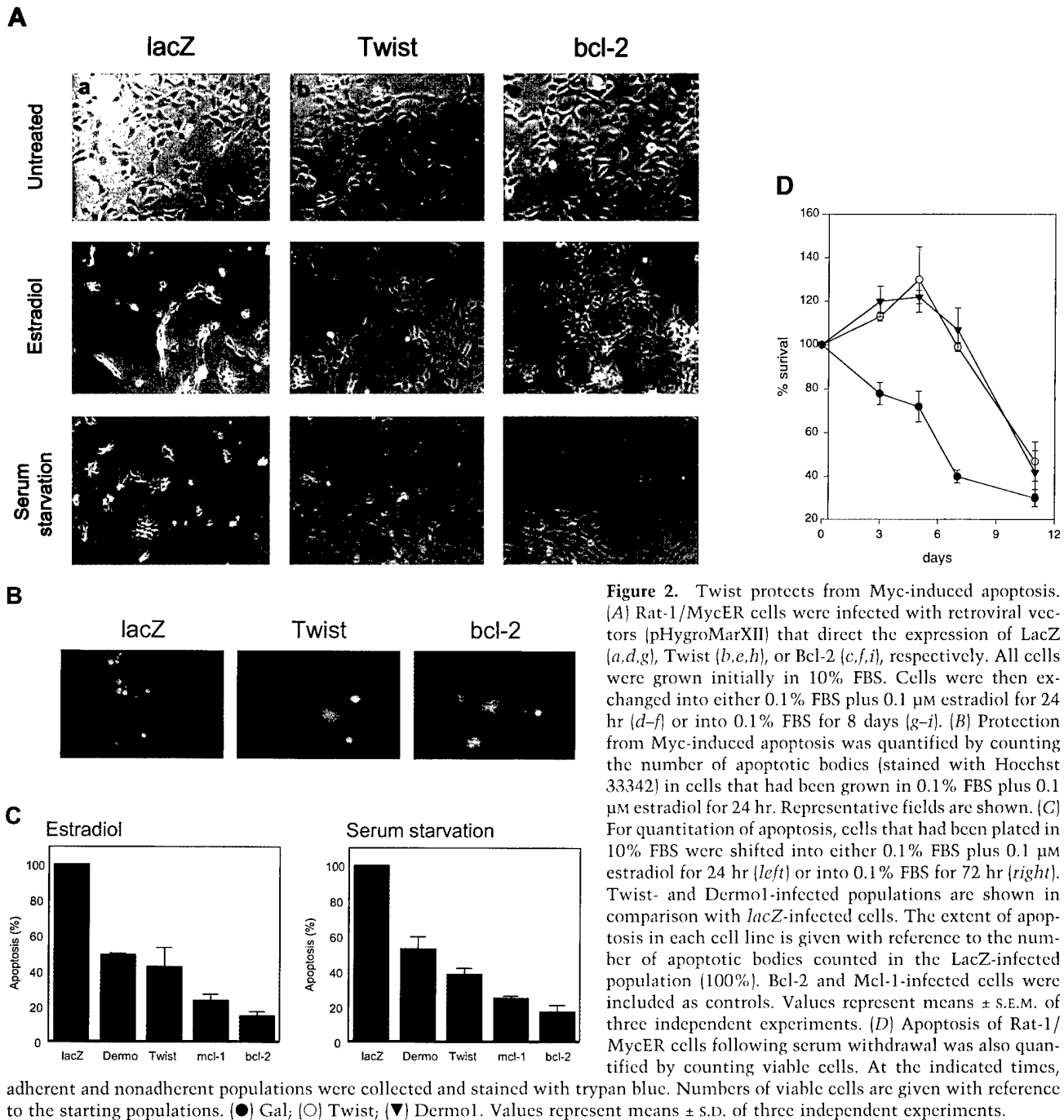
The enrichment of two such closely related proteins during the genetic selection and the isolation of each from multiple, independent cell pools prompted a deeper investigation of the possibility that Twist and Dermo1 could interfere with oncogene-induced apoptosis. Expression of either Twist or Dermo1 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



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

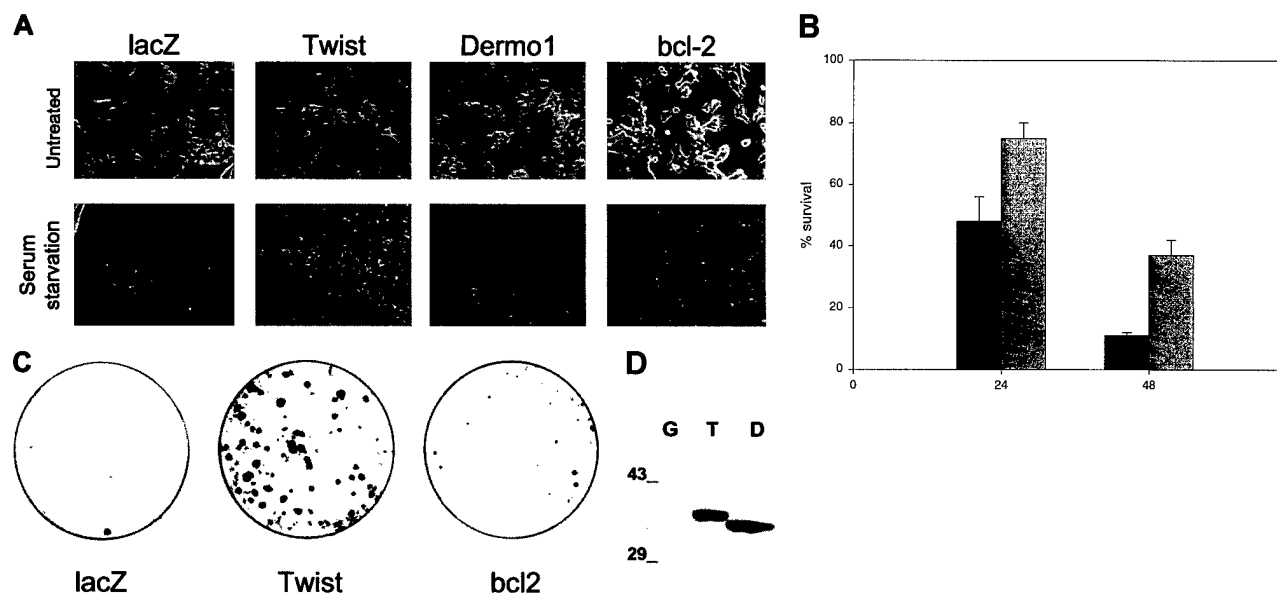


**Figure 3.** Twist antagonizes p53. (A) E1A-Ras expressing MEFs (C8 MEFs) were infected with a retroviral vector (pBabe-puro) encoding LacZ, Twist, Dermo1, or Bcl-2, as indicated. Cells were plated in the presence of 10% FBS and after 24 hr were shifted to 0.1% FBS for 5 days. The result of a representative experiment is shown. Similar results were obtained after adriamycin-treatment (0.2  $\mu$ g/ml) and after contact inhibition. (B) C8 MEFs infected with retroviruses that direct the expression of either Twist (shaded bars) or  $\beta$ -gal (solid bars) were treated with 0.2  $\mu$ g/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  $\pm$  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<sub>1</sub> 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  $\beta$ -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-

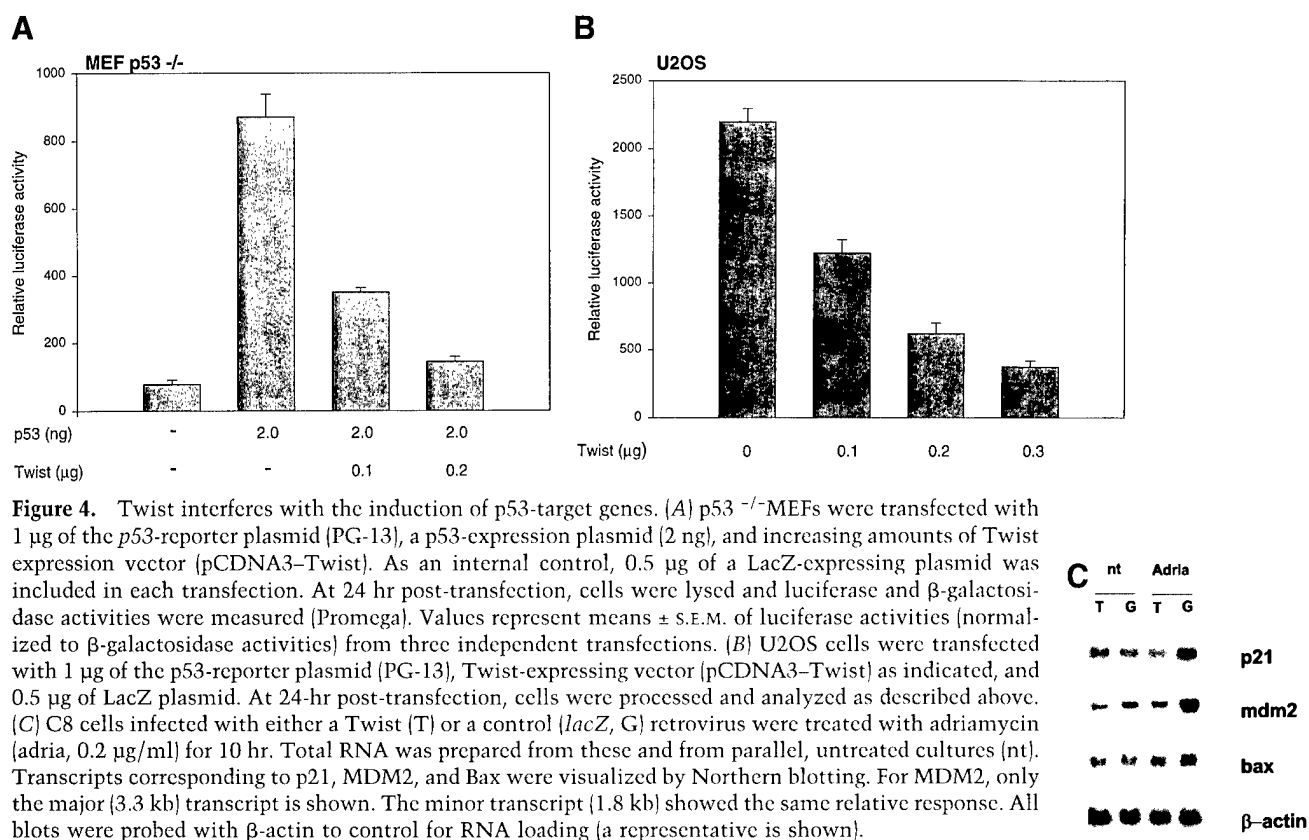


**Figure 4.** Twist interferes with the induction of p53-target genes. (A) p53<sup>-/-</sup>MEFs were transfected with 1 µg of the p53-reporter plasmid (PG-13), a p53-expression plasmid (2 ng), and increasing amounts of Twist expression vector (pCDNA3-Twist). As an internal control, 0.5 µg of a LacZ-expressing 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

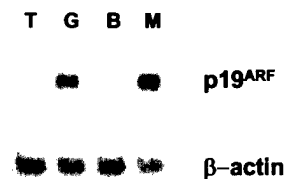


**Figure 5.** Twist down-regulates ARF. RNA was prepared from C8 cells infected with retroviruses that direct the expression of either Twist (T) or LacZ (G). ARF transcript was visualized by Northern blotting with an ARF-specific probe (exon 1β). For comparison, RNA was also prepared from BALBc 3T3 cells (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. Expres-

sion 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 (archivaly preserved primary patient material) were tested for the abundance of Twist protein. Consistent with studies on mouse embryos (Futchbauer 1995; Gitelman 1997), antibodies raised

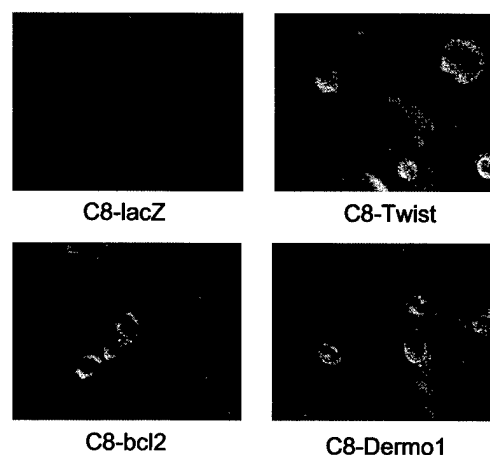


**Figure 6.** Twist and *Dermo1* promote colony formation in soft agar. C8 MEFs were infected with retroviruses that direct the expression of *LacZ*, Twist, *Dermo1*, or *Bcl-2*. Infected cells were plated in soft agar, and colony formation was assessed after 2 weeks (as indicated). The *LacZ*-expressing 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 path-

way, the loss of which is one of the most common genetic alterations in human tumors.

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<sub>2</sub> 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<sub>2</sub> 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<sup>135</sup>. 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<sub>2</sub> in DMEM supplemented with 0.01% Na pyruvate and 10% FBS at 39°C.

### Library construction and screen strategy

Poly(A)<sup>+</sup> 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*RI and *Xho*I sites (Hannon et al. 1999). The library was divided into 100 independent DNA plasmid pools, each with a complexity of 10<sup>4</sup>–10<sup>5</sup> clones. Each plasmid pool was used to transfect LinX E packaging cells by the

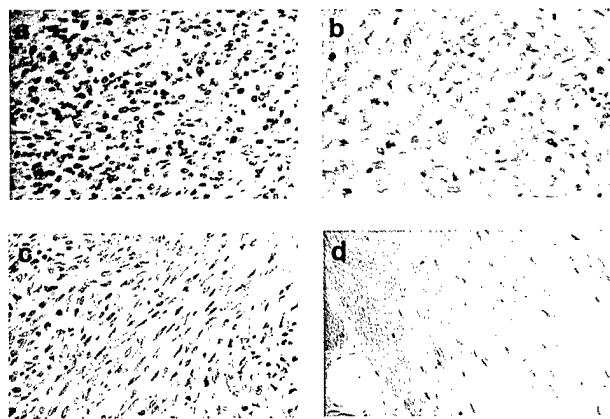


**Figure 7.** Twist is highly expressed in rhabdomyosarcomas. (a) Formalin-fixed histologic sections of a rhabdomyosarcoma were stained with a Twist-specific antibody (SC-6070). Most of the neoplastic cells show nuclear accumulation of Twist protein. (b) Specificity of the signal was confirmed by loss 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 $\times$ .

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 hygromycin and then subjected to an apoptosis/rescue schedule as described: A total of  $5 \times 10^5$  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, Dermol, 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 µl/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, Dermol, or Myc-tagged versions of Twist and Dermol. In all assays, Myc-tagged Twist and Dermol were indistinguishable from the untagged proteins. Twist and Dermol 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  $-20^\circ\text{C}$  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  $^{32}\text{P}$ -labeled probes specific for mouse *p21*, *mdm2*, *p19<sup>ARF</sup>* (exon 18), and human *bax* genes. A probe specific for mouse  $\beta$ -actin was used to confirm equal loading. Membranes were hybridized overnight at  $65^\circ\text{C}$  in 0.2 M NaPO<sub>4</sub>, 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  $60^\circ\text{C}$ , followed by autoradiography.

#### *Bypass of p53-induced growth arrest*

MEF-A3 cells that express a temperature-sensitive version of p53 (p53val<sup>135</sup>) were infected with pBABE-Puro vectors that drive the expression of LacZ, Twist, or Bcl-2, respectively. After selection,  $5 \times 10^4$  cells were plated in quadruplicate in 10 cm plates. On the following day, two plates were shifted to the permissive temperature ( $32^\circ\text{C}$ ). 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<sup>-/-</sup> 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  $\beta$ -galactosidase activities were measured by a MLX microtiter plate luminometer (Dynex, Chantilly, VA). Values represent means  $\pm$  S.E.M. of luciferase activities (normalized to  $\beta$ -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, Dermol, 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.

## Acknowledgments

R.M. was supported by a grant from the Italian Association for Cancer Research (AIRC). L.K. is supported in part by a grant from the National Institutes of Health (NIH). Y.H. is supported by an Initial Investigatorship (1104-F11) and V.S. by a grant-in-aid (1060-G1) from the American Heart Association of Greater Los Angeles. D.B. is the Hugh and Catherine Stevenson Chair in Cancer Biology. G.J.H. is supported by grants from the US Army (DAMD 17-96-1-6053), the NIH, and the Stewart Trust and is a Pew Scholar in the Biomedical Sciences. We thank Chris McCollough, Sara Piccinin, and Martina Fabris for their help and support, Bert Vogelstein for providing the PG-13 reporter construct, and Scott Lowe and Linda Penn for C8 MEF and Rat-1/MycER cells, respectively.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked 'advertisement' in accordance with 18 USC section 1734 solely to indicate this fact.

## References

- Agarwal, M.L., W.R. Taylor, M.V. Chernov, O.B. Chernova, and G.R. Stark. 1998. The p53 network. *J. Biol. Chem.* **273**: 1-4.
- Attardi, L.D., S.W. Lowe, J. Brugarolas, and T. Jacks. 1996. Transcriptional activation by p53, but not induction of the p21 gene, is essential for oncogene-mediated apoptosis. *EMBO J.* **15**: 3693-3701.
- Avantaggiati, M.L., V. Ogryzko, K. Gardner, A. Giordano, A.S. Levine, and K. Kelly. 1997. Recruitment of p300/CBP in p53-dependent signal pathways. *Cell* **89**: 1175-1184.
- Bates, S., A.C. Phillips, P.A. Clark, F. Stott, G. Peters, R.L. Ludwig, and K.H. Vousden. 1998. p14ARF links the tumor suppressors RB and p53. *Nature* **395**: 124-125.
- Bourez, R.L., I.M. Mathijssen, J.M. Vaandrager, and C. Vermeij-Keers. 1997. Apoptotic cell death during normal embryogenesis of the coronal suture: Early detection of apoptosis in mice using annexin V. *J. Craniofac. Surg.* **8**: 441-445.
- Chen, X., L.J. Ko, L. Jayaraman, and C. Prives. 1996. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. *Genes & Dev.* **10**: 2438-2451.
- Chen, Z.F. and R.R. Behringer. 1995. *twist* is required in head mesenchyme for cranial neural tube morphogenesis. *Genes & Dev.* **9**: 686-699.
- Debbas, M. and E. White. 1993. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes & Dev.* **7**: 546-554.
- DeGregori, J., G. Leone, A. Miron, L. Jokoi, and J.R. Nevins. 1997. Distinct roles for E2F proteins in cell growth control and apoptosis. *Proc. Natl. Acad. Sci.* **94**: 7245-7250.
- Deiss, L.P. and A. Kimchi. 1991. A genetic tool used to identify thioredoxin as a mediator of a growth inhibitory signal. *Science* **252**: 117-120.
- De Stanchina, E., M.E. McCurrach, F. Zindy, S.-Y. Shieh, G. Ferbeyre, A.V. Samuelson, C. Prives, M.F. Roussel, C.J. Sherr, and S.W. Lowe. 1998. E1A signaling to p53 involves the p19ARF tumor suppressor. *Genes & Dev.* **12**: 2434-2442.
- el Ghouzzi, V., M. Le Merrer, F. Perrin-Schmitt, E. Lajeunie, P. Benit, D. Renier, P. Bourgeois, A.L. Bolcato-Bellemin, A. Munnich, and J. Bonaventure. 1997. Mutations of the TWIST gene in the Saethre-Chotzen syndrome. *Nat. Genet.* **15**: 42-46.
- Evan, G.I., A.H. Wyllie, C.S. Gilbert, T.D. Littlewood, H. Land, M. Brooks, C.M. Waters, L.Z. Penn, and D.C. Hancock. 1992. Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69**: 119-128.
- Fuchtbauer, E.M. 1995. Expression of M-twist during postimplantation development of the mouse. *Dev. Dyn.* **204**: 316-322.
- Gitelman, I. 1997. Twist protein in mouse embryogenesis. *Dev. Biol.* **189**: 205-214.
- Grossman, S.R., M. Perez, A.L. Kung, M. Joseph, C. Mansur, Z. Xiao, S. Kumar, P.M. Howley, and D.M. Livingston. 1998. p300/MDM2 complexes participate in MDM2-mediated p53 degradation. *Mol. Cell* **2**: 405-415.
- Gu, W., X.L. Shi, and R.G. Roeder. 1997. Synergistic activation of transcription by CBP and p53. *Nature* **387**: 819-823.
- Gudkov, A.V. and I.B. Roninson. 1997. Isolation of genetic suppressor elements (GSEs) from random fragment cDNA libraries in retroviral vectors. *Methods Mol. Biol.* **69**: 221-240.
- Gudkov, A.V., A.R. Kazarov, R. Thimmapaya, S.A. Axenovich, I.A. Mazo, and I.B. Roninson. 1994. Cloning mammalian genes by expression selection of genetic suppressor elements: Association of kinesin with drug resistance and cell immortalization. *Proc. Natl. Acad. Sci.* **91**: 3744-3748.
- Hamamori, Y., W. Hung-Yi, V. Sartorelli, and L. Kedes. 1997. The basic domain of myogenic basic helix-loop-helix (bHLH) proteins is the novel target for direct inhibition by another bHLH protein, Twist. *Mol. Cell. Biol.* **17**: 6563-6573.
- Hamamori, Y., V. Sartorelli, V. Ogryzko, P.L. Puri, H.Y. Wu, J.Y. Wang, Y. Nakatani, and L. Kedes. 1999. Regulation of histone acetyltransferases p300 and PCAF by the bHLH protein twist and adenoviral E1A. *Cell* **96**: 405-413.
- Hannon, G.J., D. Demetrick, and D. Beach. 1993. Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. *Genes & Dev.* **7**: 2378-2391.
- Hannon, G.J., P. Sun, A. Carniero, L.Y. Xie, R. Maestro, D. Conklin, and D. Beach. 1999. Genetics in mammalian cells. *Science* **283**: 1129-1130.
- Haupt, Y., R. Maya, A. Kazaz, and M. Oren. 1997. Mdm2 promotes the rapid degradation of p53. *Nature* **387**: 296-299.
- Hebrok, M., A. Fuchtbauer, and E.M. Fuchtbauer. 1997. Repression of muscle-specific gene activation by the murine Twist

- protein. *Exp. Cell Res.* **232**: 295–303.
- Hermeking, H. and D. Eick. 1994. Mediation of c-Myc-induced apoptosis by p53. *Science* **265**: 2091–2093.
- Hockenbery, D.M., Z.N. Oltvai, X.M. Yin, C.L. Millman, and S.J. Korsmeyer. 1993. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75**: 241–251.
- Honda, R. and H. Yasuda. 1999. Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *EMBO J.* **18**: 22–27.
- Howard, T.D., W.A. Paznekas, E.D. Green, L.C. Chiang, N. Ma, R.I. Ortiz de Luna, C. Garcia Delgado, M. Gonzalez-Ramos, A.D. Kline, and E.W. Jabs. 1997. Mutations in TWIST, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome. *Nat. Genet.* **15**: 36–41.
- Kamijo, T., J.D. Weber, G. Zambetti, F. Zindy, M.F. Roussel, and C.J. Sherr. 1998. Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. *Proc. Natl. Acad. Sci.* **95**: 822–829.
- Kauffmann-Zeh, A., P. Rodriguez-Vician, E. Ulrich, C. Gilbert, P. Coffey, J. Downward, and G. Evan. 1997. Suppression of c-Myc-induced apoptosis by Ras signaling through PI3K and PKB. *Nature* **385**: 544–548.
- Kimchi, A. 1998. DAP genes: Novel apoptotic genes isolated by a functional approach to gene cloning. *Biochim. Biophys. Acta* **1377**: F13–F33.
- Ko, L.J. and C. Prives. 1996. p53: Puzzle and paradigm. *Genes & Dev.* **10**: 1054–1072.
- Kozopas, K.M., T. Yang, H.L. Buchan, P. Zhou, and R.W. Craig. 1993. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proc. Natl. Acad. Sci.* **90**: 3516–3520.
- Kubbutat, M.H., S.N. Jones, and K.H. Vousden. 1997. Regulation of p53 stability by Mdm2. *Nature* **278**: 261–263.
- Li, L., P. Cserjesi, and E.N. Olson. 1995. Dermo-1: A novel twist-related bHLH protein expressed in the developing dermis. *Dev. Biol.* **172**: 280–292.
- Lill, N.L., S.R. Grossman, D. Ginsberg, J. DeCaprio, and D.M. Livingston. 1997. Binding and modulation of p53 by p300/CBP coactivators. *Nature* **387**: 823–827.
- Lin, A.W., M. Barradas, J.C. Stone, L. van Aelst, M. Serrano, and S.W. Lowe. 1998. Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes & Dev.* **12**: 3008–3019.
- Lowe, S.W. and H.E. Ruley. 1993. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes & Dev.* **7**: 535–545.
- Lowe, S.W., H.E. Ruley, T. Jacks, and D.E. Housman. 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* **74**: 957–967.
- Lowe, S.W., T. Jacks, D.E. Housman, and H.E. Ruley. 1994. Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells. *Proc. Natl. Acad. Sci.* **91**: 2026–2030.
- Michalovitz, D., O. Halevy, and M. Oren. 1990. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell* **62**: 671–680.
- Nikiforov, M.A., K. Hagen, V.S. Ossovskaya, T.M. Connor, S.W. Lowe, G.I. Deichman, and A.V. Gudkov. 1996. p53 modulation of anchorage independent growth and experimental metastasis. *Oncogene* **13**: 1709–1719.
- Pappo, A.S. 1996. Rhabdomyosarcoma and other soft tissue sarcomas in children. *Curr. Opin. Oncol.* **8**: 311–316.
- Quelle, D.E., F. Zindy, R.A. Ashmun, and C.J. Sherr. 1995. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* **83**: 993–1000.
- Rayner, J.R. and T.J. Gonda. 1994. A simple and efficient procedure for generating stable expression libraries by cDNA cloning in a retroviral vector. *Mol. Cell. Biol.* **14**: 880–887.
- Robertson, K.D. and P.A. Jones. 1998. The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. *Mol. Cell. Biol.* **18**: 6457–6473.
- Serrano, M., A.W. Lin, M.E. McCurrach, D. Beach, and S.W. Lowe. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**: 593–602.
- Shieh, S.Y., M. Ikeda, Y. Taya, and C. Prives. 1997. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* **91**: 325–334.
- Siciliano, J.D., C.E. Canman, Y. Taya, K. Sakaguchi, E. Appella, and M.B. Kastan. 1997. DNA damage induces phosphorylation of the amino terminus of p53. *Genes & Dev.* **11**: 3471–3481.
- Soengas, M.S., R.M. Alarcon, H. Yoshida, A.J. Giaccia, R. Hakem, T.W. Mak, and S.W. Lowe. 1999. Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science* **284**: 156–159.
- Spicer, D.B., J. Rhee, W.L. Cheung, and A.B. Lassar. 1996. Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. *Science* **272**: 1476–1480.
- Sun, P., P. Dong, K. Dai, G.J. Hannon, and D. Beach. 1998. p53-independent role of MDM2 in TGF- $\beta$  resistance. *Science* **282**: 2270–2272.
- Thisse, B., M. el Messal, and F. Perrin-Schmitt. 1987. The twist gene: Isolation of a *Drosophila* zygotic gene necessary for the establishment of dorsoventral pattern. *Nucleic Acids Res.* **15**: 3439–3453.
- Vaux, D.L., S. Cory, and J.M. Adams. 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* **335**: 440–442.
- Vogelstein, B. and K.W. Kinzler. 1998. *The genetic basis of human cancer*. McGraw-Hill, New York, NY.
- Wagner, A.J., J.M. Kokontis, and N. Hay. 1994. Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21waf1/cip1. *Genes & Dev.* **23**: 2817–2830.
- Weber, J.D., L.J. Taylor, M.F. Roussel, C.J. Sherr, and D. Bar-Sagi. 1999. Nucleolar Arf sequesters Mdm2 and activates p53. *Nat. Cell Biol.* **1**: 20–26.
- Wong, B.Y., H. Chen, S.W. Chung, and P.M. Wong. 1994. High-efficiency identification of genes by functional analysis from a retroviral cDNA library. *J. Virol.* **68**: 5523–5531.
- Zhang, Y. and Y. Xiong. 1999. Mutations in human ARF exon 2 disrupt its nucleolar localization and impair its ability to block nuclear export of MDM2 and p53. *Mol. Cell* **3**: 579–591.
- Zhang, Y., Y. Xiong, and W.G. Yarbrough. 1998. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* **92**: 725–734.
- Zindy, F., C.M. Eischen, D.H. Randle, T. Kamijo, J.L. Cleveland, C.J. Sherr, and M.R. Roussel. 1998. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes & Dev.* **12**: 2424–2433.

## A Proinflammatory Cytokine Inhibits p53 Tumor Suppressor Activity

By James D. Hudson,\* Mahmood A. Shoaibi,\* Roberta Maestro,<sup>†</sup> Amancio Carnero,\* Gregory J. Hannon,<sup>§</sup> and David H. Beach\*

From the \*Unit of Cancer Biology, Institute of Child Health, London WC1N 1EH, United Kingdom; <sup>†</sup>Experimental Oncology 1, Centro di Riferimento Oncologico, 33081 Aviano, Italy; and <sup>§</sup>Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

### 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

**E**lucidating 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). Tu-

mor 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).<sup>1</sup> 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<sup>-/-</sup> Mouse Embryonic Fibroblast Cell Line.* p53<sup>-/-</sup> mouse embryonic fibroblasts (MEFs; from T.

<sup>1</sup>Abbreviations used in this paper: FBS, fetal bovine serum; GFP, green fluorescent protein; GSNO, S-nitrosoglutathione; MBP, maltose binding protein; MEF, mouse embryonic fibroblast; MIF, macrophage migration inhibitory factor; NO, nitric oxide; SNP, sodium nitroprusside.

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 a 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  $\mu$ g/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  $\sim$ 5,000 cells/plate. 1  $\mu$ g/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  $\sim$ 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-p53 $\alpha$ s, 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  $\sim$ 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 Rat-1/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  $\mu$ M 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 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  $\mu$ g/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 Axioptot 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  $\mu$ g) 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  $\Delta$ p21; 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  $\mu$ g was separated in a 1% formaldehyde gel and blotted to Hybond-N<sup>+</sup> 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 phosphor/fluorescent imager, and normalized to loaded RNA by quantification of fluorescence of ethidium bromide-stained ribosomal RNA bands in 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- $\beta$ -gal, a plasmid which carries  $\beta$ -galactosidase under the control of the CMV promoter. 1 d after transfection, cells were split, pooled, and replated at  $\sim$ 500,000 cells/plate. 150 ng/ml rMIF was added to half of the plates. The next day, 1  $\mu$ g/ml doxycycline was added to the media to induce GFP-p53. At 0 and 10 h after induction, extracts were prepared, and luciferase and  $\beta$ -galactosidase activities were assayed using Promega kits. Luciferase reporter activities were normalized to  $\beta$ -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<sup>-/-</sup> 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 excision after in-

tegration 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  $4 \times 10^6$  cells were infected. After drug selection for the library vector, cells were split at varying dilutions, and 1  $\mu\text{g}/\text{ml}$  doxycycline was added to the media to induce expression of 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. Provirus containing cDNAs from positive clones were recovered by Hirt extraction.

Provirus 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 (Rat-1/mycER) were infected with pools of a cDNA library prepared from Rat-1/mycER cells committed to apoptosis in pHygroMarx II. After drug selection, cells were induced to undergo apoptosis by shifting to low serum media (DMEM + 0.1% FBS) plus 0.1  $\mu\text{M}$  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. Provirus 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 the presence or absence of recombinantly produced MIF (rMIF) and doxycycline. Colony formation was observed

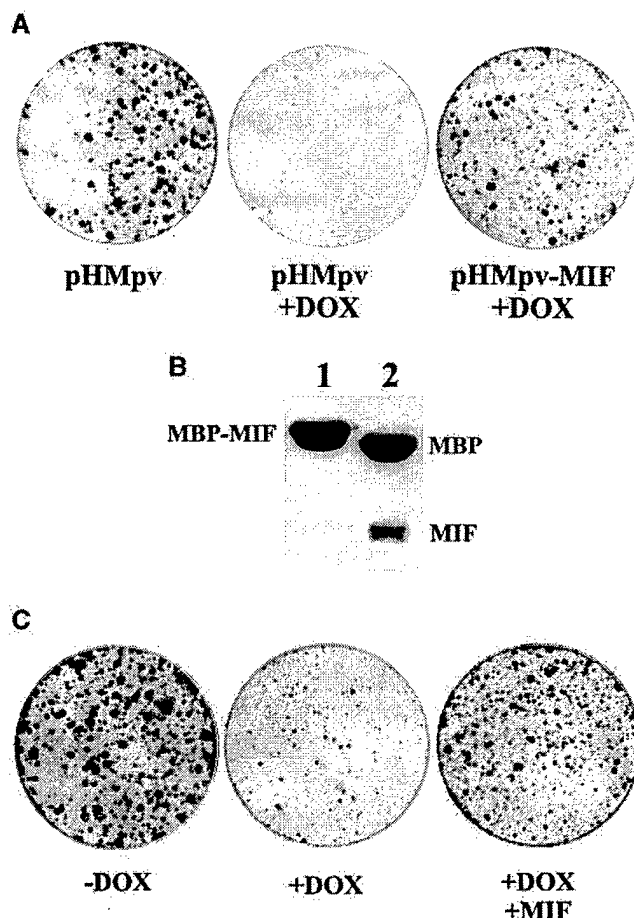


**Figure 1.** MIF treatment overcomes p53-induced growth arrest. (A) Expression of MIF bypasses p53-induced growth arrest and allows colony formation in a tetracycline-inducible GFP-p53 cell line. pH Mpv, HygroMarx I-based provirus; HMpv-MIF, HygroMarx I-based provirus expressing human MIF; DOX, 1  $\mu\text{g}/\text{ml}$  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.

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).

p53 primarily functions via its ability to transactivate gene expression. Therefore, we tested whether MIF treat-

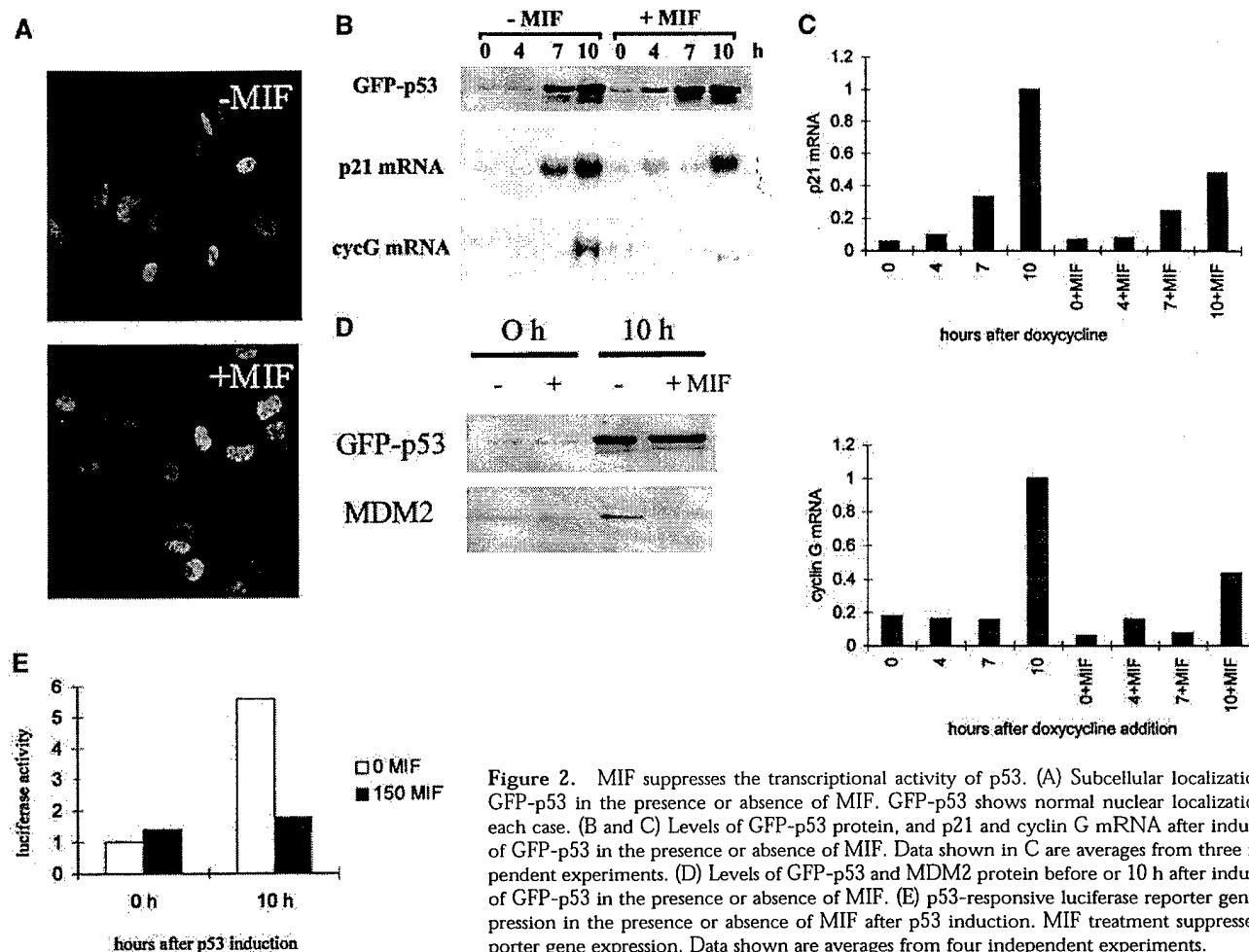


**Figure 2.** MIF suppresses the transcriptional activity of p53. (A) Subcellular localization of GFP-p53 in the presence or absence of MIF. GFP-p53 shows normal nuclear localization in each case. (B and C) Levels of GFP-p53 protein, and p21 and cyclin G mRNA after induction of GFP-p53 in the presence 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.

ment 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 with 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 suscepti-

ble 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 dependent (36, 37). To test whether MIF treatment was capable of suppressing NO-induced apoptosis, we treated RAW264.7 macrophages with NO-releas-

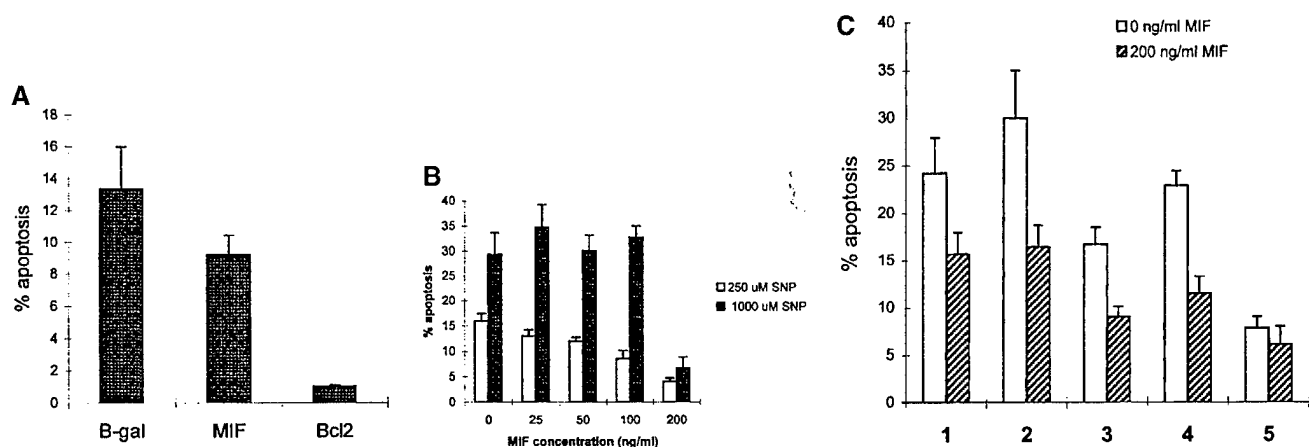


**Figure 3.** MIF treatment overcomes p53-dependent apoptosis in fibroblasts and macrophages. (A) Apoptosis in Rat-1/mycER cells expressing LacZ, MIF, or Bcl2 cDNAs were shifted to media containing 0.1% FBS plus 0.1  $\mu$ M estradiol to induce apoptosis. After 24 h, cells were stained with Hoechst 33342 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  $\mu$ M 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.

ers, 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  $2\text{--}3 \times 10^{-4}$  and  $1\text{--}3 \times 10^{-3}$  with fibroblasts prepared from a p53<sup>-/-</sup> 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

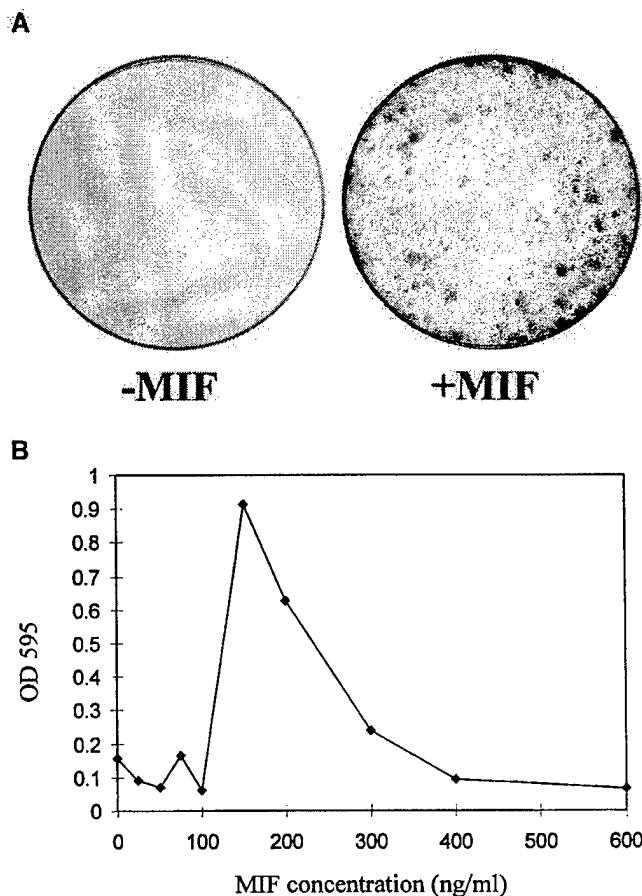


**Figure 4.** (A) Primary MEFs show extended life span in the presence of 200 ng/ml rMIF. Cells one passage before senescence were plated in the presence and absence of MIF, and stained after 15 d. Numerous colonies were formed only in the presence of MIF. (B) Dose dependency of MIF treatment in inducing 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.

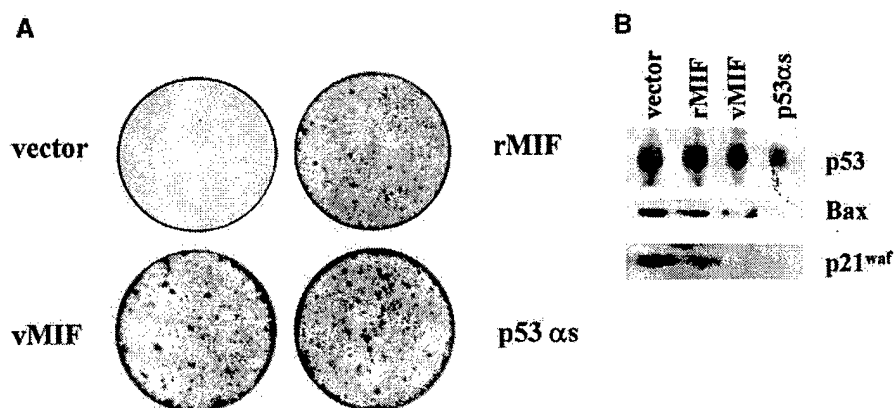


**Figure 5.** MIF biological activity correlates with suppression of p53-mediated target gene expression. Primary MEFs expressing MIF (vMIF), an antisense directed against p53 (p53 $\alpha$ s), control vector (vector), or treated with rMIF were plated in passage 5. 15 d after plating cells were (A) fixed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

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 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.

We thank Lin Xie for the use of LinX retrovirus producer cells, D. Conklin for the use of the A431 cDNA library in pHygroMarx I, and Michela Armellini for her assistance in scoring apoptotic cells. Many thanks to P. Otavio de Campos Lima, P. Sun, R. Levinsky, and D. Conklin for helpful discussions and additional reagents.

This work was supported by a grant from the Cancer Research Campaign (to J. Hudson and D. Beach). J. Hudson was supported by a grant from the Leukaemia Research Fund. A. Carnero was supported by an EMBO long-term fellowship. R. Maestro was supported by a grant from the Italian Association for Cancer Research. G. Hannon is a Pew Scholar in the Biomedical Sciences. D. Beach is supported by the Hugh and Catherine Stevenson Fund.

Address correspondence to David Beach, Institute of Child Health, University College London, Cruciform Building, Gower Street, London WC1E 6BT, UK. Phone: 171-813-8495; Fax: 171-813-0358; E-mail: D.Beach@ich.ucl.ac.uk

Submitted: 26 July 1999 Accepted: 5 August 1999

## References

1. Ko, L.J., and C. Prives. 1996. p53: puzzle and paradigm. *Genes Dev.* 10:1054-1072.
2. Hansen, R., and M. Oren. 1997. p53: from inductive signal to cellular effect. *Curr. Opin. Genet. Dev.* 7:46-51.
3. Maltzman, W., and L. Czyzyk. 1981. UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. *Mol. Cell Biol.* 4:1689-1694.
4. Kastan, M.B., O. Onyekwere, D. Sidransky, B. Vogelstein, and R.W. Craig. 1991. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.* 51:6304-6311.
5. Yonish-Rouach, E., D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi, and M. Oren. 1991. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature.* 353:345-347.
6. Kuerbitz, S.J., B.S. Plunkett, W.V. Walsh, and M.B. Kastan. 1992. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci. USA.* 89:7491-7495.
7. Clarke, A.R., C.A. Purdie, D.J. Harrison, R.G. Morris, C.C. Bird, M.L. Hooper, and A.H. Wyllie. 1993. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature.* 362:849-852.
8. Lowe, S.W., E.M. Schmitt, S.W. Smith, B.A. Osborne, and T. Jacks. 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature.* 362:847-849.
9. Lane, D.P. 1992. Cancer. p53, guardian of the genome. *Nature.* 358:15-16.
10. Griffiths, S.D., A.R. Clarke, L.E. Healy, G. Ross, A.M. Ford, M.L. Hooper, A.H. Wyllie, and M. Greaves. 1997. Absence of p53 permits propagation of mutant cells following genotoxic damage. *Oncogene.* 14:523-531.
11. Hayflick, L., and P.S. Moorhead. 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* 25:585-621.
12. Jenkins, J.R., K. Rudge, and G.A. Currie. 1984. Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. *Nature.* 312:651-654.
13. Shay, J.W., O.M. Pereira-Smith, and W.E. Wright. 1991. A role for both RB and p53 in the regulation of human cellular senescence. *Exp. Cell Res.* 196:33-39.
14. Harvey, M., A.T. Sands, R.S. Weiss, M.E. Hegi, R.W. Wiseman, P. Pantazis, B.C. Giovanella, M.A. Tainsky, A. Bradley, and L.A. Donehower. 1993. In vitro growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene.* 8:2457-2467.
15. Moll, U.M., G. Riou, and A.J. Levine. 1992. Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. *Proc. Natl. Acad. Sci. USA.* 89:7262-7266.
16. Moll, U.M., M. LaQuaglia, J. Benard, and G. Riou. 1995. Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. *Proc. Natl. Acad. Sci. USA.* 92:4407-4411.
17. Maestro, R., A. Gloghini, C. Doglioni, D. Gasparotto, T. Vukosavljevic, V. De Re, L. Laurino, A. Carbone, and M. Boiocchi. 1995. MDM2 overexpression does not account for stabilization of wild-type p53 protein in non-Hodgkin's lymphomas. *Blood.* 85:3239-3246.
18. Gordon, L.I., and S.A. Weitzman. 1990. Inflammation and cancer: role of phagocyte-generated oxidants in carcinogenesis. *Blood.* 76:655-663.
19. Christen, S., T.M. Hagen, M.K. Shigenaga, and B.N. Ames. 1999. Chronic infection and inflammation lead to cancer. In *Microbes and Malignancy: Infection as a Cause of Cancer*. J. Parsonnet and S. Horning, editors. Oxford University Press, Oxford.
20. Weitberg, A.B., S.A. Weitzman, M. Destrempe, S.A. Latt, and T.P. Stossel. 1983. Stimulated human phagocytes produce cytogenetic changes in cultured mammalian cells. *N. Engl. J. Med.* 308:26-30.
21. Weitzman, S.A., A.B. Weitberg, E.P. Clark, and T.P. Stossel. 1985. Phagocytes as carcinogens: malignant transformation produced by human neutrophils. *Science.* 227:1231-1233.
22. Gossen, M., S. Freundlieb, G. Bender, G. Muller, W. Hillen, and H. Bujard. 1995. Transcriptional activation by tetracyclines in mammalian cells. *Science.* 268:1766-1769.
23. Brugarolas, J., C. Chandrasekaran, J.I. Gordon, D. Beach, T. Jacks, and G.J. Hannon. 1995. Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature.* 377:552-557.
24. Norris, P.S., and M. Haas. 1997. A fluorescent p53GFP fusion protein facilitates its detection in mammalian cells while retaining the properties of wild-type p53. *Oncogene.* 15:2241-2247.
25. Hannon, G.J., P.-Q. Sun, A. Carnero, L. Xie, R. Maestro, D.S. Conklin, and D.H. Beach. 1999. MarX: an approach to genetics in mammalian cells. *Science.* 283:1129-1130.
26. Calandra, T., and R. Bucala. 1997. Macrophage migration inhibitory factor (MIF): a glucocorticoid counter-regulator within the immune system. *Crit. Rev. Immunol.* 17:77-88.
27. Maestro, R., A.P. Deitos, Y. Hamamori, S. Krasnokutsky, V. Sartorelli, L. Kedes, C. Doglioni, D. Beach, and G. Hannon. 1999. *twist* is a potential oncogene that inhibits apoptosis. *Genes Dev.* 13:2207-2217.
28. Wagner, A.J., J.M. Kokontis, and N. Hay. 1994. Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21<sup>waf1/cip1</sup>. *Genes Dev.* 8:2817-2830.

29. El-Deiry, W.S., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell*. 75:817-825.
30. Harper, J.W., G.R. Adami, N. Wei, K. Keyomarsi, and S.J. Elledge. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*. 75: 805-816.
31. Xiong, Y., G.J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach. 1993. p21 is a universal inhibitor of cyclin kinases. *Nature*. 366:701-704.
32. Okamoto, K., and D. Beach. 1994. Cyclin G is a transcriptional target of the p53 tumor suppressor protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4816-4822.
33. Barak, Y., T. Juven, R. Haffner, and M. Oren. 1993. mdm2 expression is induced by wild type p53 activity. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:461-468.
34. Wu, X., J.H. Bayle, D. Olson, and A.J. Levine. 1993. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev.* 7:1126-1132.
35. El-Deiry, W.S., S.E. Kern, J.A. Pietenpol, K.W. Kinzler, and B. Vogelstein. 1992. Definition of a consensus binding site for p53. *Nat. Genet.* 1:45-49.
36. Messmer, U.K., and B. Brune. 1996. Nitric oxide (NO) in apoptotic versus necrotic RAW 264.7 macrophage cell death: the role of NO-donor exposure, NAD<sup>+</sup> content, and p53 accumulation. *Arch. Biochem. Biophys.* 327:1-10.
37. Messmer, U.K., and B. Brune. 1996. Nitric oxide-induced apoptosis: p53-dependent and p53-independent signalling pathways. *Biochem. J.* 319:299-305.
38. Miyashita, T., and J.C. Reed. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell*. 80:293-299.
39. Bacher, M., C.N. Metz, T. Calandra, K. Mayer, J. Chesney, M. Lohoff, D. Gerns, T. Donnelly, and R. Bucala. 1996. An essential regulatory role for macrophage migration inhibitory factor in T-cell activation. *Proc. Natl. Acad. Sci. USA*. 93: 7849-7854.
40. Onodera, S., K. Suzuki, T. Matsuno, K. Kaneda, M. Takagi, and J. Nishihira. 1997. Macrophage migration inhibitory factor induces phagocytosis of foreign particles by macrophages in autocrine and paracrine fashion. *Immunology*. 92:131-137.
41. MacMicking, J., Q.W. Xie, and C. Nathan. 1997. Nitric oxide and macrophage function. *Annu. Rev. Immunol.* 15:323-350.
42. Schaffer, C.J., and L.B. Nanney. 1996. Cell biology of wound healing. *Int. Rev. Cytol.* 169:151-181.
43. Krishna, M., B. Woda, L. Savas, S. Baker, and B. Banner. 1995. Expression of p53 antigen in inflamed and regenerated mucosa in ulcerative colitis and Crohn's disease. *Mod. Pathol.* 8:654-657.
44. Hibi, K., H. Mitomi, W. Koizumi, S. Tanabe, K. Saigenji, and I. Okayasu. 1997. Enhanced cellular proliferation and p53 accumulation in gastric mucosa chronically infected with *Helicobacter pylori*. *Am. J. Clin. Pathol.* 108:26-34.
45. Wotherspoon, A.C. 1998. *Helicobacter pylori* infection and gastric lymphoma. *Br. Med. Bull.* 54:79-85.

# Loss-of-function genetics in mammalian cells: the p53 tumor suppressor model

Amancio Carnero, James D. Hudson, Gregory J. Hannon<sup>1</sup> and David H. Beach\*

Institute of Child Health, 30 Guilford Street, London WC1 1EH, UK and <sup>1</sup>Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA

Received February 18, 2000; Revised and Accepted April 7, 2000

## ABSTRACT

Using an improved system for the functional identification of active antisense fragments, we have isolated antisense fragments which inactivate the p53 tumour suppressor gene. These antisense fragments map in two small regions between nt 350 and 700 and nt 800 and 950 of the coding sequence. These antisense fragments appear to act by inhibition of p53 mRNA translation both *in vivo* and *in vitro*. Expression of these antisense fragments overcame the p53-induced growth arrest in a cell line which expresses a thermolabile mutant of p53 and extended the *in vitro* lifespan of primary mouse embryonic fibroblasts. Continued expression of the p53 antisense fragment contributed to immortalisation of primary mouse fibroblasts. Subsequent elimination of the antisense fragment in these immortalised cells led to restoration of p53 expression and growth arrest, indicating that immortal cells continuously require inactivation of p53. Expression of MDM2 or SV40 large T antigen, but not E7 nor oncogenic ras, overcomes the arrest induced by restoration of p53 expression. Functional inactivation of both p21 and bax (by overexpression of Bcl2), but not either alone, allowed some bypass of p53-induced growth arrest, indicating that multiple transcriptional targets of p53 may mediate its antiproliferative action. The ability to conditionally inactivate and subsequently restore normal gene function may be extremely valuable for genetic analysis of genes for which loss-of-function is involved in specific phenotypes.

## INTRODUCTION

Human tumours are caused by the progressive accumulation of activating mutations in oncogenes and loss of function mutations in tumour suppressor genes. A subset of tumour suppressor genes function to prevent tumour formation or growth by inducing cell cycle arrest or apoptosis in response to potentially oncogenic events. For example, tumour suppressor genes

such as p53 and Rb encode proteins that are activated and restrain proliferation following expression of activated alleles of oncogenes, such as H-ras (1–3).

Indeed, p53 is the most commonly mutated gene in human cancer (reviewed in 4–7), underscoring its importance in the suppression of tumour formation. p53 functions as a transcription factor which plays an important role in the maintenance of genomic stability. Following genotoxic damage, p53 is induced and acts to restrain proliferation by inducing the expression of genes which lead to growth arrest (such as p21) or apoptosis (such as bax). By inhibiting proliferation following DNA damage, p53 action prevents the accumulation of potentially oncogenic mutations.

p53 also functions to suppress cellular immortalisation (8–11). Normal primary somatic cells are capable of undergoing a finite number of divisions in culture until they undergo cellular senescence, characterised by growth arrest, a large flat morphology and insensitivity to further mitogenic stimulation (reviewed in 12–14). In contrast, many tumour cells exhibit unlimited division potential, indicating that they have bypassed the barriers to immortalisation, such as cellular senescence. p53 appears to play a direct role in controlling the onset of cellular senescence. p53 transcriptional activity increases with ageing of the cells (15), wild-type p53 activity is necessary for growth arrest in senescence (11,16,17) and a high percentage of cells that escape from senescence have lost p53 activity (18–21).

Numerous antitumour therapeutic strategies have been proposed based on the ability to restore wild-type p53 function by refolding of mutant protein or relocalisation of mislocalised protein (22,23). However, while p53 is among the best studied tumour suppressor genes to date, it is not yet clear whether continual p53 inactivation is necessary for continued expression of the immortalisation phenotype, i.e. whether re-expression of wild-type levels of p53 is sufficient to halt the proliferation of immortalised cells with different genetic backgrounds. This must be true for such therapeutic strategies to be successful. The ability to conditionally inhibit tumour suppressor function and subsequently restore wild-type levels of gene expression under normal physiological control is essential to answer this question and can also be used to examine functional relationships between genes in mammalian cells. While a variety of techniques are available to inhibit gene function, such as genetic disruptions

\*To whom correspondence should be addressed at present address: Wolfson Institute for Biomedical Research, UCL, Cruciform Building, Gower Street, London WC1E 6BT, UK. Tel: +44 171 679 6771; Fax: +44 171 209 0470; Email: d.beach@ucl.ac.uk

Present address:

Amancio Carnero, Wolfson Institute for Biomedical Research, UCL, Cruciform Building, Gower Street, London WC1E 6BT, UK

in mice and somatic cell gene disruptions in human cultured cells, these techniques are effectively not reversible. An alternative approach which is applicable to a wide variety of cell types and is potentially reversible is the delivery or expression of antisense constructs.

The most common approach to targeted gene inhibition in mammalian cells involves the intracellular expression of antisense RNA complementary to the mRNA of the target gene (24,25). Antisense RNA techniques offer a powerful tool for down-regulating the expression of specific genes both *in vitro* and *in vivo* (26–33). A number of molecular mechanisms have been proposed to explain the action of antisense RNAs (34). For example, sense–antisense RNA duplexes can form in the nucleus where they are rapidly degraded, improperly processed or not transported to the cytosol in cultured cells (30). In transient systems or when antisense RNA is injected into the cytoplasm, the antisense RNA may act by binding to the mRNA and inhibiting its translation (35).

Despite the ease of expressing antisense RNA for any cloned gene, many antisense RNA constructs have little or no biological effect (36–38). Some of the more effective antisense RNAs comprise only a part of the coding sequence and there are no clear rules to predict which portion of the mRNA will give rise to the most effective antisense inhibitor (38–41).

We have used a systematic approach to select antisense RNA fragments which are active in inhibiting the function of the p53 tumour suppressor gene. These antisense fragments are highly effective in repressing p53 protein expression and biological function. Reversible expression of these antisense fragments has been used to demonstrate the role of p53 in immortalisation and shed light on its functional relationship to other immortalisation genes. The ability to conditionally inactivate and subsequently restore normal gene function may be extremely valuable for the analysis of the function of a wide range of genes; this methodology could be more widely applied to provide insight into the function of other antiproliferative genes.

## MATERIALS AND METHODS

### Generation of the mouse p53 antisense library

mRNA was transcribed *in vitro* from pBluescript-p53 using T7 RNA polymerase (Stratagene). After RNA synthesis, the product was treated with RNase-free DNase (10 U/ $\mu$ g DNA) for 30 min at 37°C and phenol extracted. RNA was electrophoresed on a 4% acrylamide–7 M urea gel and p53 mRNA was purified. Aliquots of 2  $\mu$ g of p53 mRNA were used in generating the library. Randomly primed cDNA fragments of the p53 gene were synthesised, size selected (50–500 nt) on a S400 column (Pharmacia) and cloned into the *Eco*RI and *Xho*I sites of pMARXIVpuro in the antisense orientation.

### Cell culture and preparation of mouse embryo fibroblasts (MEFs)

MEFs were prepared from day 13.5 embryos derived from CD1 mice. The head and blood organs were removed, then the torso was minced and dispersed in 0.1% trypsin (45 min at 37°C). Cells were grown for two population doublings and then frozen. MEFs were subcultured 1:4 upon reaching confluence; each passage was considered to be two population doubling levels (PDs). p53–/– MEFs were obtained from embryos derived

from crosses between p53+/- mice. To create a cell line expressing temperature-sensitive p53, immortalised p53–/– MEFs were infected with pWZL-p53Val135. Clones were isolated at the permissive temperature (39°C) and a clone was selected that showed highly efficient growth arrest following a temperature shift to the restrictive temperature (32°C). All cultures were maintained in DMEM (Gibco) plus 10% foetal bovine serum (Sigma). Where necessary, cells were selected when indicated with 75  $\mu$ g/ml hygromycin (Calbiochem), 400  $\mu$ g/ml G418 (Sigma) or 4  $\mu$ g/ml puromycin (Fluka).

### 3T3 immortalisation protocols

MEFs were infected and selected as before. Every 3 days, cells were trypsinised, counted and  $10^6$  cells were plated per 10 cm plate.

### Retroviral-mediated gene transfer

Samples of  $5 \times 10^6$  LinXE ecotropic retrovirus producer cells were plated per 10 cm dish, incubated for 24 h and transfected by calcium phosphate precipitation using 20  $\mu$ g of retroviral plasmid. After 48 h, the virus-containing medium was filtered (0.45  $\mu$ m filter; Millipore) and supplemented with 8  $\mu$ g/ml polybrene (Sigma) and an equal volume of fresh medium. One day before infection, target fibroblasts were plated at  $8 \times 10^5$  cells/10 cm dish and incubated overnight. For infections, culture medium was replaced by the appropriate viral supernatant and the culture plates were centrifuged (1 h, 1500 r.p.m.) and incubated at 37°C for 16 h.

### *In vitro* recovery of the proviruses

Total genomic DNA was extracted from cells from a confluent 10 cm plate. It was then treated with RNase A (50  $\mu$ g/ml, 30 min) and proteinase K (100  $\mu$ g/ml final concentration) and extracted twice with phenol/chloroform. Following ethanol precipitation, genomic DNA was washed extensively with 70% ethanol and dissolved in 200  $\mu$ l of ultrapure water.

To excise the proviruses, 10  $\mu$ g of genomic DNA was digested with CRE recombinase (DNA final concentration 0.1  $\mu$ g/ $\mu$ l) for 3 h at 37°C, extracted with phenol/chloroform and ethanol precipitated. DNA was washed extensively with 70% ethanol and dissolved in 5  $\mu$ l of water. Aliquots of 2–5 mg of total DNA were electroporated into DH10B-lac-trfA bacteria and proviruses recovered from zeocin-resistant bacterial colonies.

### Immunoblot analysis

Cells were washed twice with ice-cold PBS and lysed in lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris–HCl, pH 8.0, 1 mM PMSF, 1  $\mu$ g/ml leupeptin, 25  $\mu$ g/ml aprotinin, 1 mM EDTA). After 15 min on ice, lysates were vortexed (5 min at 4°C) and cleared by centrifugation. Aliquots of 100–150  $\mu$ g of total protein (Bio-Rad protein assay) were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. Western blot analysis was carried out using standard procedures and detected using ECL (Amersham). Ab1-421 (Oncogene Research) was used to detect mouse p53; antibodies 240 and 246 (Santa Cruz) were used to detect mutant and wild-type p53 protein by immunoprecipitation. An affinity-purified rabbit polyclonal antibody was used for p21<sup>WAF</sup>, followed by detection with horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse antibodies (Amersham).

### Northern blot

Total RNA was isolated from subconfluent cultures (Trizol; Gibco BRL). Ten micrograms of RNA was resolved by electrophoresis, transferred to Hybond-N<sup>+</sup> membranes and probed according to standard procedures. p53 mRNA was detected using a radiolabelled fragment containing the entire coding sequence of mouse p53.

### In vitro translation

*In vitro* translation was performed in rabbit reticulocyte lysates (TNT T7-T3; Promega) following the vendor's instructions. Mouse p53 was transcribed and translated from pBlueScript-p53 under control of the T7 promoter. Where necessary, p53 antisense fragments cloned into pBlueScript under the control of the T3 promoter were included in the reaction.

## RESULTS

### Isolation of p53 antisense fragments

We have tested our system against the p53 gene that has been used previously as the target in similar approaches (42,43). To obtain antisense fragments which inhibited p53 activity, we undertook a phenotype-based screen to directly identify fragments which inhibited p53 gene function. We first generated clones of immortalised fibroblasts derived from p53-disrupted mice which expressed a thermolabile p53 mutant (p53ts p53<sup>-/-</sup> MEF). Clones grew normally at a permissive temperature (39°C) at which p53 is inactive, but arrested when shifted to a restrictive temperature (32°C) where p53 adopts the wild-type conformation (44).

A random antisense fragment library directed against the coding sequence of a mouse p53 library was constructed and cloned into the MMLV-based retroviral vector pMARXIVpuro (45). This library contained ~10<sup>4</sup> independent clones. Library DNA was transfected into ecotropic retrovirus packaging cells and replication-deficient viruses were infected into exponentially growing p53ts p53<sup>-/-</sup> MEF cells. Following selection for puromycin resistance, ~10<sup>5</sup> cells/100 mm culture dish were plated and shifted to 32°C. After 3 weeks, approximately 30 colonies were formed at 32°C, indicating that they had overcome the growth inhibitory action of p53. These colonies were subcloned and grown at 32°C for an additional 2 weeks. After this second round of phenotypic selection, 17 colonies continued to grow.

Genomic DNA was extracted from positive colonies and proviruses containing the antisense fragment were excised. A total of 19 different constructs were recovered. The majority of the colonies carried a single provirus. Fourteen of the 19 constructs had a p53-derived fragment in the antisense orientation (Table 1).

The active antisense fragments localised to two small clusters between nt 350 and 700 (2/14) and between nt 800 and 950 (12/14). Among these antisense fragments, number 2 was recovered six times from independent colonies. Sequencing of representatives of the unselected library showed that the fragments were randomly distributed over the p53 coding sequence (data not shown), indicating that the selection for these specific regions was a property of the phenotypic screen.

**Table 1.** p53 antisense fragments recovered from a phenotypic screen in p53ts p53<sup>-/-</sup> MEF cells

| Cell clone | Provirus recovered | Length | Sequence  | Assigned number |
|------------|--------------------|--------|-----------|-----------------|
| 2          | p53 antisense      | 141    | 706–565   | 15              |
| 3          | p53 antisense      | 90     | 937–847   | 61              |
| 4          | p53 antisense      | 114    | 920–806   | 2               |
| 5          | p53 antisense      | 113    | 906–793   | 57              |
|            | p53 antisense      | 120    | 923–803   | 55              |
| 6          | p53 antisense      | 87     | 923–836   | 71              |
| 7          | p53 antisense      | 114    | 920–806   | 2               |
| 10         | p53 antisense      | 273    | 670–357   | 76              |
| 11         | empty vector       |        |           |                 |
| 12         | Tandem (sense)     | 120    | 1065–1185 | 53              |
| 13         | p53 antisense      | 114    | 920–806   | 2               |
| 14         | p53 antisense      | 40     | 940–900   | 13              |
|            | p53 antisense      | 114    | 920–806   | 2               |
| 15         | Empty primers      |        |           |                 |
| 16         | p53 antisense      | 114    | 920–806   | 2               |
| 17         | p53 antisense      | 114    | 920–806   | 2               |
| 18         | Empty primers      |        |           |                 |
| 19         | p53 antisense      | 121    | 911–790   | 17              |
| 21         | Empty vector       |        |           |                 |

### Biological properties of p53 antisense fragments

The effectiveness of these antisense fragments was confirmed by reinfected p53ts p53<sup>-/-</sup> MEF cells with positive proviruses. Following drug selection, cells were plated at 5 × 10<sup>4</sup> cells/100 mm dish and shifted to 32°C. Control cells with the vector alone did not grow at the restrictive temperature (Fig. 1) and exhibited a flat enlarged cellular morphology (data not shown). In contrast, cells expressing the p53 antisense fragments showed a high rate of colony formation at this temperature; fragments derived from the first region (number 15) showed less penetrance than those derived from the second region (numbers 55, 71, 12 and 2). Most of the cells expressing the p53 antisense fragments were small, refractive and did not cease proliferation at subconfluent densities (data not shown). Thus, selected p53 antisense fragments were effective in inhibiting the p53-induced arrest.

We next tested the effect of antisense expression on p53 protein levels. Following infection of p53ts p53<sup>-/-</sup> MEF cells and drug selection, cells were lysed and levels of p53 protein were analysed. While expression of each of the antisense fragments reduced p53 protein levels, antisense fragments derived from the second region were more effective (Fig. 2A).

Variations in penetrance of individual antisense constructs within a population might result from differences in expression levels. We therefore infected p53ts p53<sup>-/-</sup> MEF cells with different antisense fragments, shifted cells to 32°C and after 3 weeks selected p53-resistant colonies. Two different clones expressing each of the antisense sequences numbers 15, 55, 71 and 2 were expanded and p53 levels were analysed (Fig. 2B).

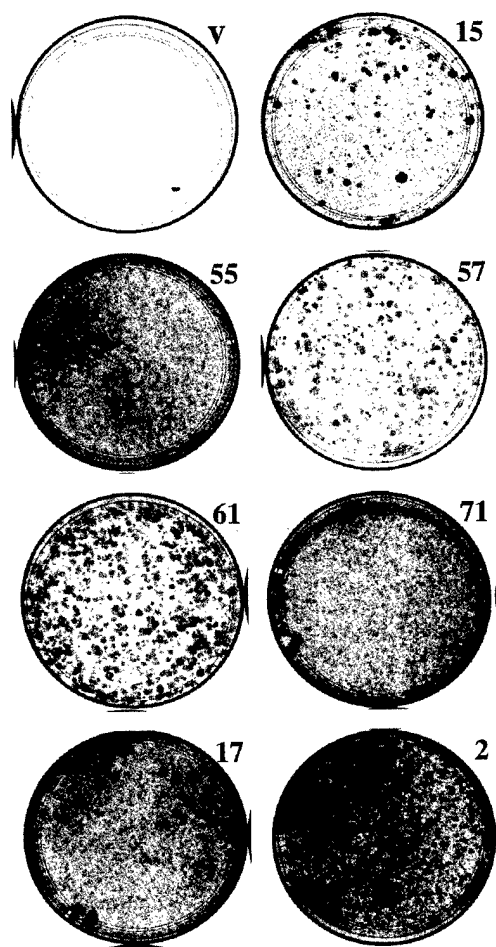


**Figure 1.** Expression of p53 antisense fragments overcomes p53-induced growth arrest. p53ts p53<sup>-/-</sup> MEF cells were infected with viruses that expressed p53 antisense fragments (numbers 15, 55, 57, 61, 71, 17 and 2) or empty vector (v). After drug selection for virally transduced cells,  $5 \times 10^4$  cells were plated in 10 cm dishes and grown at restrictive temperature (32°C) for 15 days. Cells were then fixed and stained with crystal violet.

p53 protein was only detectable in colonies expressing antisense fragment number 15, consistent with its less penetrant ability to induce colony formation and inhibit p53 accumulation in mass culture. In each case, the level of p53 protein in the selected clones was lower than in the mass culture, indicating that there was selection for clones in which the antisense fragments were more active.

p53 functions via its activity as a transcription factor by inducing expression of growth arrest-inducing genes such as p21 (46). To test whether antisense fragment expression interfered with p53 activity we analysed levels of p21 protein in cells that had been infected with antisense-expressing or control vectors. Following a shift to 32°C control cells arrested and induced expression of p21, while colonies derived from p53 antisense selection were unable to induce p21 under similar conditions, indicating that reduced p53 activity was correlated with decreased protein expression following antisense expression (Fig. 2B).

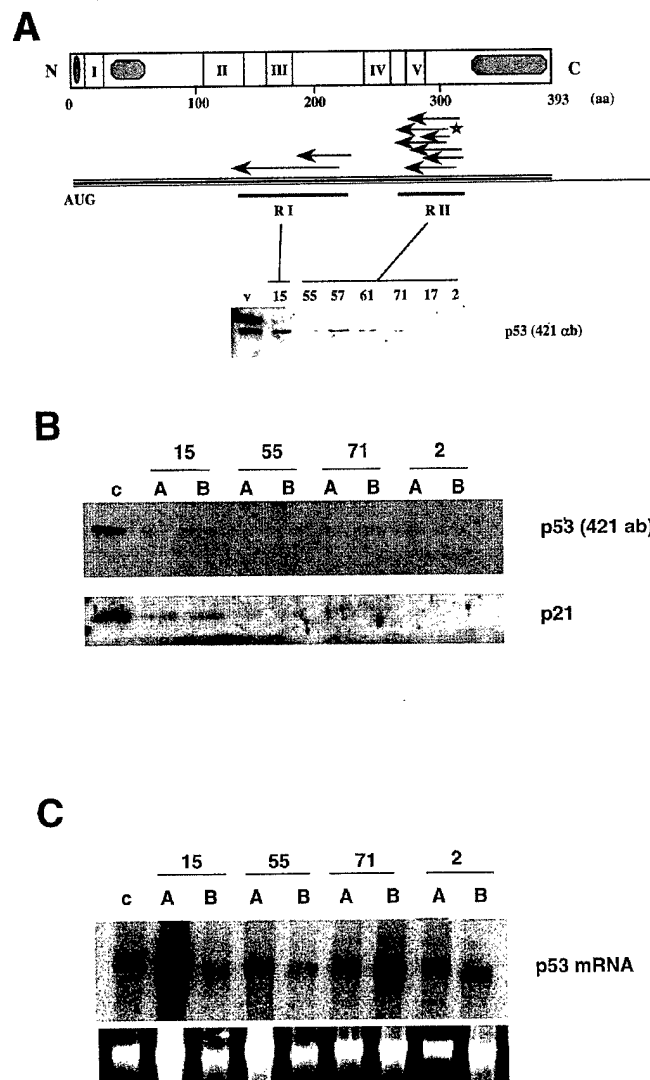


**Figure 2.** Localisation and activity of p53 antisense fragments. (A) p53 antisense fragments isolated following phenotypic selection mapped to two regions, RI and RII, of the p53 coding sequence. Expression of these antisense fragments reduces p53 protein levels *in vivo*. p53ts p53<sup>-/-</sup> MEF cells were infected with viruses expressing different antisense fragments (numbers 15, 55, 57, 61, 71, 17 and 2) or control vector (v). Infectants were selected, cells were lysed and p53 protein levels were assayed by western blot. (B) Expression of p53 antisense fragments reduces the expression of p21, a transcriptional target of p53. Two clones (A and B) of antisense-expressing p53ts p53<sup>-/-</sup> MEF cells (numbers 15, 55, 71 and 2) or vector alone (c) were selected and shifted to 32°C for induction of p53 activity. After 24 h, cells were lysed and levels of p53 or p21 protein were analysed by western blot. (C) Expression of p53 antisense fragments does not affect levels of p53 mRNA *in vivo*. Northern blot of p53 mRNA in colonies expressing p53 antisense fragments. Total RNA was prepared from antisense-expressing clones as above, separated by gel electrophoresis, transferred to Hybond membranes and probed with a radiolabelled p53-specific probe (Upper). (Lower) Ethidium bromide stained 28S band of the total RNA. The results of densitometric analysis of p53 versus 28S mRNA bands showed no significant variation with respect to control cells (not shown).

To try to understand the mechanism by which the antisense fragments inhibited p53 expression, we next tested whether antisense fragment expression interfered with p53 mRNA

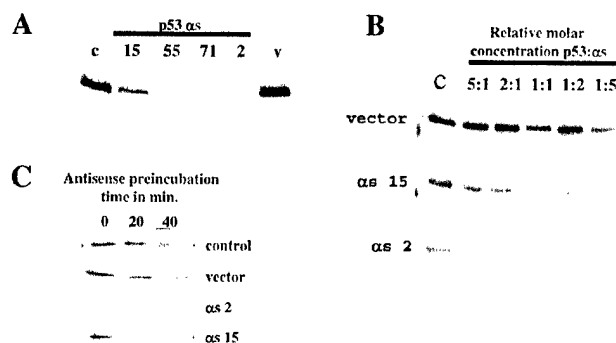


**Figure 3.** Inhibition of p53 translation by expression of p53 antisense fragments *in vitro*. (A) p53 protein was translated *in vitro* in the presence of p53 antisense fragment transcripts (numbers 15, 55, 71 and 2), control vector (v) or no additional vector (c). (B) *In vitro* translation of p53 protein in the presence of different molar ratios of p53 antisense transcripts (numbers 15 and 2) or control vector. (C) Effect of preincubation of antisense transcripts on translation of p53 protein.

expression. Antisense fragment expression had no detectable effect upon p53 mRNA expression (Fig. 2C), indicating that the p53 antisense fragments did not function via a mechanism involving mRNA degradation.

To further elaborate on the mechanism of antisense fragment action, we analysed the effect of RNA antisense expression in a cell-free system. Mouse p53 was transcribed using T7 polymerase and translated in reticulocyte lysates (Fig. 3). In the same reaction, antisense constructs or control vectors were expressed under control of the T3 promoter. Expression of p53 antisense fragments greatly reduced (number 15) or almost completely abolished (numbers 55, 71 and 2) p53 translation (Fig. 3A). Levels of inhibition *in vitro* correlate with those found *in vivo* (Fig. 2), indicating that the p53 antisense fragments might act by inhibiting translation of p53 protein. To assess whether the different effects of the antisense fragments might be due to differences in the efficiency of translational inhibition we translated p53 in the presence of increasing molar concentrations of antisense fragments derived from the first (number 15) and second (number 2) regions of p53 (Fig. 3D). In each case antisense number 2 is more efficient than number 15 in inhibiting translation, however, increasing levels of each improves their relative efficiency of inhibition (Fig. 3C and D).

These results are consistent with a mechanism in which the antisense fragments interfered with p53 protein synthesis rather than degradation of the RNA duplex or blocking of transport across the nuclear membrane, although this last mechanism cannot be excluded *in vivo*.

#### Biological effect of p53 antisense fragments in primary fibroblasts

We next tested the ability of the p53 antisense fragments to interfere with wild-type p53 function in primary cells. Specifically, we tested whether expression of p53 antisense fragments, like loss of p53 function, was capable of extending the *in vitro* lifespan of primary MEFs. MEFs in passage 3 were infected with viruses expressing p53 antisense fragments or control vector. Infected cells were selected and cultured until passage

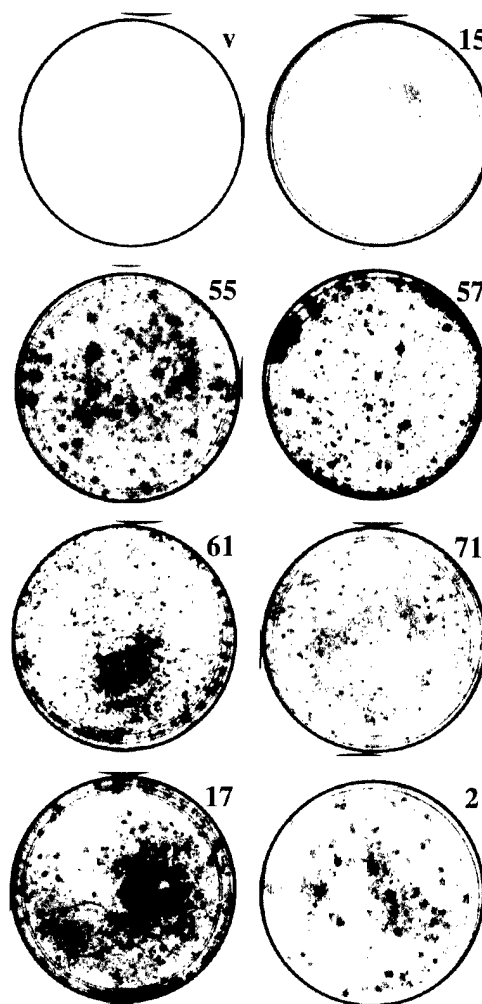


**Figure 4.** Expression of p53 antisense fragments induces an extended lifespan of primary MEFs. Primary MEFs were infected with antisense-expressing viruses (numbers 15, 55, 57, 61, 71, 17 and 2) or control vector (v) in PD 2. After selection, cells were passaged until PD 12, at which time  $10^5$  cells were plated in 10 cm dishes and grown for 15 days. Cells were then fixed and stained with crystal violet. Cells which exhibit an extended lifespan are capable of colony formation under these conditions.

5, the passage immediately prior to the passage at which wild-type MEFs undergo cellular senescence. Cells were plated at  $3 \times 10^5$ /100 mm dish and cultured for 15 days. Under these conditions wild-type cells do not form colonies, however, cells that display an extended cellular lifespan can continue to divide and can form colonies (16,47). MEFs infected with vector alone displayed a flat phenotype and grew very poorly. Surprisingly, the same phenotype was observed in cells expressing antisense fragment 15. In contrast, cells which expressed p53 antisense fragments derived from the second region were able to grow and form colonies (Fig. 4).

We also carried out a similar functional screen to identify p53 antisense fragments which were capable of directly inducing an extended lifespan in primary MEFs. From this screen we isolated five antisense fragments, all of which

mapped to region II of the p53 gene (data not shown), independently confirming the effectiveness of fragments derived from this region.

We next tested the relative efficiency of the p53 antisense fragments in producing an extended lifespan. Expression of antisense fragments showed a similar penetrance in inducing colony formation as expression of full-length antisense or dominant negative p53 (175H) or expression of HPV E7 (which inactivates members of the Rb family). However, they did not produce as extended a lifespan as that of MEFs derived from a p53-disrupted mouse (Table 2).

**Table 2.** Relative penetrance of p53 inactivation in inducing an extended lifespan of primary MEFs

| MEF genotype       | Relative penetrance  |
|--------------------|----------------------|
| Wild type          | 0                    |
| p53 <sup>-/-</sup> | $3.3 \times 10^{-2}$ |
| p53 $\alpha$ s     | $4 \times 10^{-3}$   |
| p53(175H)          | $4 \times 10^{-3}$   |
| E7                 | $3 \times 10^{-3}$   |

Primary MEFs were prepared from wild-type mouse embryos and infected with viruses expressing p53 antisense fragments, dominant negative p53 or E7. Murine fibroblasts were prepared from p53<sup>-/-</sup> mouse embryos for comparison. Cells were plated in PD 12, grown for 15 days, fixed and stained. Relative penetrance was measured as number of colonies formed per plated cell.

### Recovery of physiological levels of p53 expression in immortalised MEFs

We next sought to test the effect of restoration of wild-type p53 levels in immortalised cells. For these experiments, we cloned the p53 antisense fragments into pMarxIV (45), a retroviral vector that contains a CRE recombinase target site (loxP site). Upon integration of the retrovirus into the genome, the loxP site is duplicated such that the genes carried by the virus are flanked on either side by loxP sites. Subsequent expression of CRE recombinase causes excision at these loxP sites, leading to removal from the genome and eventual loss of the construct. Therefore, gene expression in pMarxIV is effectively reversible. Thus, antisense constructs directed against p53 would be excised following CRE expression, leading to loss of the antisense fragment and restoration of physiological levels of p53 (Fig. 5A).

We took advantage of this feature to test whether continuous inactivation of p53 function was required for immortalisation. Presenescent MEFs were infected with two different p53 $\alpha$ s (numbers 2 and 71) in pMarxIV and immortal cell lines, p53 $\alpha$ s<sup>CR</sup> cells (CR for CRE-reversible) were generated using a 3T3 protocol. At PD 32, p53 $\alpha$ s<sup>CR</sup> cells were infected with a CRE-expressing virus to ablate antisense expression. Cells in which p53 function had been restored failed to form colonies, while control cells continued to proliferate (Fig. 5). As an additional control, immortalised cell lines that had been generated following infection with non-excisable p53 antisense-expressing viruses did not arrest following CRE recombinase expression

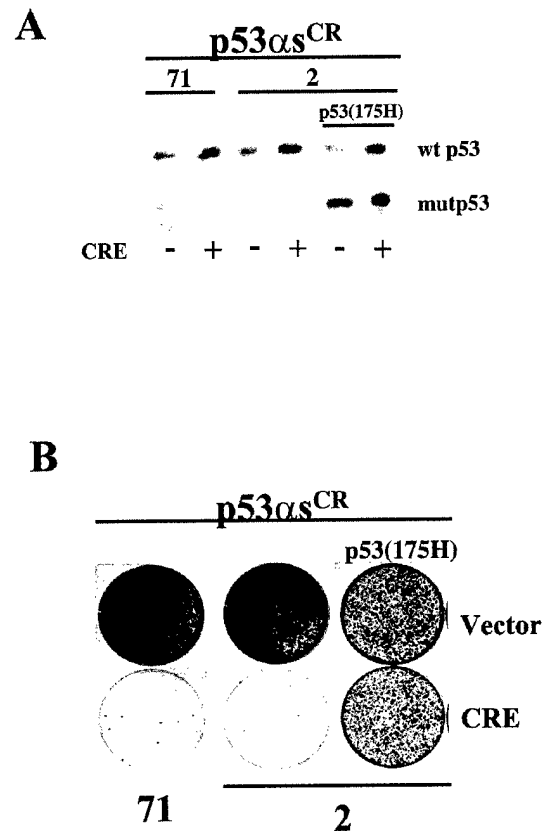


**Figure 5.** Recovery of p53 expression leads to growth arrest in immortalised cells. Presenescent MEFs were infected with viruses directing the expression of CRE-excisable pMarxIVp53 $\alpha$ s71 or pMarxIVp53 $\alpha$ s2 and immortalised using a 3T3 immortalisation protocol. Immortalised cells (p53 $\alpha$ s<sup>CR</sup>) were infected with a control vector or pWZL-Hygro-CRE to excise the integrated p53 antisense construct leading to restoration of p53 expression. p53 $\alpha$ s<sup>CR</sup> cells were infected with a virus expressing dominant negative p53, p53(175H), prior to CRE infection. (A) Levels of expression of wild-type p53 (wt p53) or mutant p53 (mutp53) in the p53 $\alpha$ s<sup>CR</sup> cells after infection with CRE or vector alone. (B) Colony formation of p53 $\alpha$ s<sup>CR</sup> cells infected with viruses carrying CRE or vector alone. Cells were plated at equal densities, cultured in the presence of hygromycin for 10–15 days, fixed and stained with crystal violet.

(data not shown). Finally, expression of dominant negative p53 from a non-excisable vector in the p53 $\alpha$ s<sup>CR</sup> reversible cell line overcame the arrest induced by excision of the p53 antisense construct, indicating that the recovery of mortality was solely dependent on p53. Together these data indicate that continuous inactivation of p53 function is required for continued immortalisation.

Having a cell line which was reversibly dependent on loss of p53 function allowed us to test whether expression or inactivation of other genes could suppress the requirement for continued p53 inactivation. We therefore tested whether expression of MDM2 (a gene which targets p53 for degradation) (48,49), SV40 large T antigen (a viral oncogene which binds to and inactivates p53 and Rb) (50), HPV E7 (a protein which inactivates members of the pRB protein family) (51) or an allele of activated H-ras (a potent cytoplasmic oncogene) (52) could bypass the growth arrest induced by re-establishment of p53 expression in

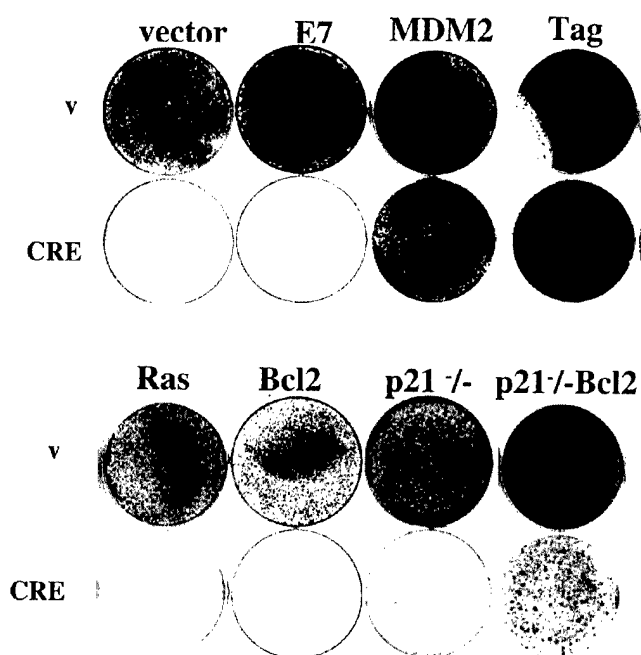


**Figure 6.** Bypass of growth arrest induced by restoration of p53 function. p53 $\alpha$ <sup>CR</sup> cells were infected with viruses directing the expression of various genes or a p53 $\alpha$ <sup>CR</sup> cell line was constructed in a p21<sup>-/-</sup> genetic background. Cells were then infected with a control or CRE-expressing virus, plated and grown for 10–15 days under selective conditions, fixed and stained with crystal violet.

p53 $\alpha$ <sup>CR</sup> cells. In this system expression of MDM2 and large T antigen, but not E7 nor H-ras, inhibited p53-induced growth arrest.

p53 presumably inhibits immortalisation by directing expression of the relevant transcriptional targets. We therefore sought to test whether inactivation of known transcriptional targets of p53, such as the antiproliferative gene p21 and pro-apoptotic gene bax, was sufficient to overcome the arrest induced by re-expression of p53 in p53 $\alpha$ <sup>CR</sup> cells. Therefore, we generated a p53 $\alpha$ <sup>CR</sup> cell line in a p21<sup>-/-</sup> genetic background or expressed Bcl2 (a gene which binds to and inactivates bax) in p53 $\alpha$ <sup>CR</sup> cells. Overexpression of Bcl2 or inactivation of p21<sup>waf1</sup> did not overcome the arrest induced by p53 recovery (Fig. 6). To test both genes simultaneously, we expressed Bcl2 in p21<sup>-/-</sup> p53 $\alpha$ <sup>CR</sup> cells. In this case, a partial bypass of the p53-dependent mortality was observed.

## DISCUSSION

Here, we have described a general strategy for identifying active antisense fragments and reversibly inactivating gene function. The identification of antisense sequences capable of suppressing target gene function has been a major goal in designing antisense vectors for gene therapy and for research use. In many cases, small antisense fragments have been more effective than full-length antisense constructs, spurring the development of methods to predict the most active antisense region. Although numerous reports describing methodologies to identify active antisense fragments exist, no consistently

reliable method has yet been described. The identification of active antisense fragments by screening random fragments on the basis of their ability to produce a biological phenotype is a direct way to identify active antisense fragments. Although functional screens for small sense or antisense gene fragments have been previously described (42,43,53–55), the method described here is optimised to specifically identify small antisense fragments and provide a simple method to recover positive fragments.

We have used this methodology to identify antisense fragments that inhibit the function of the p53 tumour suppressor. The identified fragments mapped to two regions of the p53 gene and were at least as effective as full-length p53 antisense constructs in inhibiting translation of p53 mRNA (data not shown). A previous report (42) described the isolation of p53 antisense fragments that conferred cisplatin resistance to a human ovarian adenocarcinoma cell line. They found four small active antisense fragments that mapped between nt 360 and 700 of human p53, which overlaps with region I in our study. The identification of a second region for effective p53 antisense fragments in our study may be due to the use of p53 derived from different species or to the screens not being fully saturated.

Like inactivation of p53 by genetic disruption or expression of dominant interfering alleles, the p53 antisense fragments were capable of inducing an extended lifespan in primary MEFs, although not as efficiently as knockout. Experiments showing that expression of dominant negative p53 could functionally substitute for the p53 antisense fragments clearly demonstrated the specificity of the antisense action.

Expression of the p53 antisense fragments induced an extended lifespan and contributed to the immortalisation of primary MEFs. The observation that this immortalisation was reversible upon removal of the antisense construct provides a clear demonstration that continued inactivation of p53 is necessary for immortalisation. As well, it indicates that expression of the p53 antisense fragment was sufficient to prevent selection for mutations at the p53 genomic locus during immortalisation, again confirming the efficiency and specificity of action. Reversibly immortal cell lines have been constructed using conditional expression of SV40 large T antigen (56,57). While SV40 large T antigen can bind and inactivate p53, it can also inactivate members of the Rb family as well as other unrelated proteins (50), making it difficult to determine the role of each protein in suppressing immortalisation. The use of antisense RNA provides a more specific tool to analyse the function of individual proteins.

We have constructed conditionally immortalised cell lines that are dependent on the absence of p53 function. Thus, we have created a conditional cell line in which we can restore wild-type levels of p53. Such a cell line is extremely useful for identifying and probing pathways of p53 action. The observation that expression of MDM2 or SV40 large T antigen, but not E7 nor oncogenic ras, overcomes the arrest induced by restoration of p53 expression is consistent with a role of these proteins in functionally inactivating wild-type levels of p53 (48,49). In addition, this cell line might be extremely useful in identifying the relevant transcriptional targets of p53 in preventing immortalisation. Indeed, functional inactivation of both p21 and bax, known transcriptional targets of p53, did allow some bypass of p53-induced growth arrest, indicating that multiple

transcriptional targets of p53 may mediate its antiproliferative action.

Reintroduction of missing tumour suppressors into tumours is being developed as a strategy of cancer therapy. We have shown that restoration of unaltered but inactive p53 leads to growth arrest depending on the nature of co-existing genetic alterations. p53 induces arrest in the presence of oncogenes such as ras and Bcl2, even in the absence of p21<sup>waf1</sup>. However, disturbing the p53 signal with binding oncogenes such as MDM2 and SV40 large T antigen inhibits this antitumorigenic effect, suggesting that inactivation of these proteins, when deregulated, will be more effective than reintroduction of wild-type p53.

The application of our system to a broader range of tumour suppressor genes may be useful in characterising their role in suppressing tumourigenic phenotypes.

## ACKNOWLEDGEMENTS

This work was supported by grants from the Leukaemia Research Fund (LRF 9728), Cancer Research Campaign (CRC no. SP 2366/0201 and CRC no. SP 2366/0101) and EMBO. D.H.B. was supported by the Hugh and Catherine Stevenson Fund.

## REFERENCES

- Lin, A.W., Barradas, M., Stone, J.C., van Aelst, L., Serrano, M. and Lowe, S.W. (1998) *Genes Dev.*, **12**, 3008–3019.
- Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D. and Lowe, S.W. (1997) *Cell*, **88**, 593–602.
- Xu, H.J., Zhou, Y., Ji, W., Perng, G.S., Kruzelock, R., Kong, C.T., Bast, R.C., Mills, G.B., Li, J. and Hu, S.X. (1997) *Oncogene*, **15**, 2589–2596.
- Ko, L.J. and Prives, C. (1996) *Genes Dev.*, **10**, 1054–1072.
- Lane, D.P. (1994) *Br. Med. Bull.*, **50**, 582–599.
- Steele, R.J., Thompson, A.M., Hall, P.A. and Lane, D.P. (1998) *Br. J. Surg.*, **85**, 1460–1467.
- Vogelstein, B. and Kinzler, K.W. (1992) *Cell*, **70**, 523–526.
- Foster, B.A., Coffey, H.A., Morin, M.J. and Rastinejad, F. (1999) *Science*, **286**, 2507–2510.
- Gao, Q., Hauser, S.H., Liu, X.L., Wazer, D.E., Madoc-Jones, H. and Band, V. (1996) *Cancer Res.*, **56**, 3129–3133.
- Sugrue, M.M., Shin, D.Y., Lee, S.W. and Aaronson, S.A. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 9648–9653.
- Wynford-Thomas, D., Jones, C.J. and Wyllie, F.S. (1996) *Biol. Signals*, **5**, 139–153.
- Barrett, J.C., Annab, L.A. and Futreal, P.A. (1993) *Adv. Exp. Med. Biol.*, **330**, 27–43.
- Stein, G.H. and Dulic, V. (1995) *Bioessays*, **17**, 537–543.
- Wynford-Thomas, D. (1999) *J. Pathol.*, **187**, 100–111.
- Bond, J.A., Haughton, M., Blaydes, J., Gire, V., Wynford-Thomas, D. and Wyllie, F. (1996) *Oncogene*, **13**, 2097–2104.
- Bond, J.A., Wyllie, F.S. and Wynford-Thomas, D. (1994) *Oncogene*, **9**, 1885–1889.
- Gollahon, L.S. and Shay, J.W. (1996) *Oncogene*, **12**, 715–725.
- Dalal, S., Gao, Q., Androphy, E.J. and Band, V. (1996) *J. Virol.*, **70**, 683–688.
- Finlay, C.A. (1992) *Bioessays*, **14**, 557–560.
- Harvey, D.M. and Levine, A.J. (1991) *Genes Dev.*, **5**, 2375–2385.
- Rogan, E.M., Bryan, T.M., Hukku, B., Maclean, K., Chang, A.C., Moy, E.L., Englezou, A., Warneford, S.G., Dalla-Pozza, L. and Reddel, R.R. (1995) *Mol. Cell. Biol.*, **15**, 4745–4753.
- Gallagher, W.M. and Brown, R. (1999) *Ann. Oncol.*, **10**, 139–150.
- Komarova, E.A. and Gudkov, A.V. (1998) *Semin. Cancer Biol.*, **8**, 389–400.
- Mukhopadhyay, T. and Roth, J.A. (1995) *Cancer J. Sci. Am.*, **1**, 233–248.
- Mukhopadhyay, T. and Roth, J.A. (1996) *Crit. Rev. Oncogen.*, **7**, 151–190.
- Burfeind, P., Chernicky, C.L., Rininsland, F. and Ilan, J. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 7263–7268.
- Cheng, J.Q., Ruggeri, B., Klein, W.M., Sonoda, G., Altomare, D.A., Watson, D.K. and Testa, J.R. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 3636–3641.
- Holt, J.T., Arteaga, C.B., Robertson, D. and Moses, H.L. (1996) *Hum. Gene Ther.*, **7**, 1367–1380.
- Izant, J.G. and Weintraub, H. (1985) *Science*, **229**, 345–352.
- Kim, S.K. and Wold, B.J. (1985) *Cell*, **42**, 129–138.
- Nellen, W. and Sczakiel, G. (1996) *Mol. Biotechnol.*, **6**, 7–15.
- Sokol, D.L. and Murray, J.D. (1996) *Transgenic Res.*, **5**, 363–371.
- Zhang, Y., Mukhopadhyay, T., Donehower, L.A., Georges, R.N. and Roth, J.A. (1993) *Hum. Gene Ther.*, **4**, 451–460.
- Denhardt, D.T. (1992) *Ann. NY Acad. Sci.*, **660**, 70–76.
- Melton, D.A. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 144–148.
- Kerr, S.M., Stark, G.R. and Kerr, I.M. (1988) *Eur. J. Biochem.*, **175**, 65–73.
- Leiter, J.M., Krystal, M. and Palese, P. (1989) *Virus Res.*, **14**, 141–159.
- van der Krol, A.R., Mol, J.N. and Stuitje, A.R. (1988) *Gene*, **72**, 45–50.
- Daugherty, B.L., Hotta, K., Kumar, C., Ahn, Y.H., Zhu, J.D. and Pestka, S. (1989) *Gene Anal. Tech.*, **6**, 1–16.
- Holzmayer, T.A., Pestov, D.G. and Roninson, I.B. (1992) *Nucleic Acids Res.*, **20**, 711–717.
- Rhodes, A. and James, W. (1990) *J. Gen. Virol.*, **71**, 1965–1974.
- Gallagher, W.M., Cairney, M., Schott, B., Roninson, I.B. and Brown, R. (1997) *Oncogene*, **14**, 185–193.
- Ossovskaya, V.S., Mazo, I.A., Chernov, M.V., Chernova, O.B., Strezoska, Z., Kondratov, R., Stark, G.R., Chumakov, P.M. and Gudkov, A.V. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 10309–10314.
- Martinez, J., Georgoff, I., Martinez, J. and Levine, A.J. (1991) *Genes Dev.*, **5**, 151–159.
- Hannon, G.J., Sun, P., Carnero, A., Xie, L.Y., Maestro, R., Conklin, D.S. and Beach, D. (1999) *Science*, **283**, 1129–1130.
- el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W. and Vogelstein, B. (1993) *Cell*, **75**, 817–825.
- Carnero, A., Hudson, J.D., Price, C.M. and Beach, D.H. (2000) *Nature Cell Biol.*, **2**, 148–155.
- Haupt, Y., Maya, R., Kazanietz, A. and Oren, M. (1997) *Nature*, **387**, 296–299.
- Kubbutat, M.H., Jones, S.N. and Vousden, K.H. (1997) *Nature*, **387**, 299–303.
- Pipas, J.M. (1998) *Dev. Biol. Stand.*, **94**, 313–319.
- Syrjanen, S.M. and Syrjanen, K.J. (1999) *Ann. Med.*, **31**, 175–187.
- Barbacid, M. (1987) *Annu. Rev. Biochem.*, **56**, 779–827.
- Gudkov, A.V., Kazarov, A.R., Thimmapaya, R., Axenovich, S.A., Mazo, I.A. and Roninson, I.B. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 3744–3749.
- Gudkov, A.V., Zelnick, C.R., Kazarov, A.R., Thimmapaya, R., Suttle, D.P., Beck, W.T. and Roninson, I.B. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 3231–3235.
- Roninson, I.B., Gudkov, A.V., Holzmayer, T.A., Kirschling, D.J., Kazarov, A.R., Zelnick, C.R., Mazo, I.A., Axenovich, S. and Thimmapaya, R. (1995) *Cancer Res.*, **55**, 4023–4028.
- Westerman, K.A. and Le Boulch, P. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 8971–8976.
- Wright, W.E. and Shay, J.W. (1992) *Exp. Gerontol.*, **27**, 383–389.

## brief communications

Route de Narbonne, 31062 Toulouse cedex, France  
 Institute of Cell and Molecular Biology,  
 Mayfield Road, University of Edinburgh,  
 Edinburgh EH9 3JH, UK  
 e-mail: mazars@cict.fr

1. Berridge, M. J. *Nature* **361**, 315–325 (1993).
2. Clapham, D. E. *Cell* **80**, 259–268 (1995).
3. McAnish, M. R. & Hetherington, A. M. *Trends Plant Sci.* **3**, 32–36 (1998).
4. Hardingham, G. E., Chawla, S., Johnson, C. M. & Bading, H. *Nature* **385**, 260–265 (1997).
5. Malviya, A. N. & Rogue, P. J. *Cell* **92**, 17–23 (1998).
6. Knight, M. R., Campbell, A. K., Smith, S. M. & Trevaras, A. J. *Nature* **352**, 524–526 (1991).
7. Van der Luit, A. H., Olivari, C., Halsey, A., Knight, M. R. & Trevaras, A. J. *Plant Physiol.* **121**, 705–714 (1999).
8. Takahashi, K., Isobe, M. & Muto, S. *Biochim. Biophys. Acta* **1401**, 339–346 (1998).
9. Stoppin, V., Vantard, M., Schmit, A. C. & Lambert, A. M. *Plant Cell* **6**, 1099–1106 (1994).

Cell biology

## Risky immortalization by telomerase

Senescence naturally limits the proliferation of mammalian cells in culture, possibly by shortening the telomere regions at the ends of chromosomes during cell division<sup>1,2</sup>. In support of this idea, introducing TERT, the catalytic subunit of telomerase — the enzyme that maintains chromosome ends — into certain cell types can extend their lifespan and potentially immortalize them<sup>3,4</sup>. It has been proposed that treatment with exogenous TERT might be useful for cell-based therapies by allowing indefinite expansion of normal human cells without damaging their genomes<sup>5,6</sup>. But we show here that TERT-driven cell proliferation is not genoprotective because it is associated with activation of the *c-myc* oncogene.

Human mammary epithelial cell (HMEC) cultures normally stop dividing at 55–60 population doublings (PDs). We infected these cells with a human(h)TERT retrovirus at PD40 and maintained them until PD250 (ref. 4), then tested whether telomerase activity was essential for this immortalized phenotype by excising the hTERT retrovirus at PD150 using Cre recombinase<sup>7</sup>. The resulting HMEC-Cre cells were maintained for at least another 20 population doublings and we saw no decline in growth rates in either pooled cells or individual clones. We used Southern blots of genomic DNA to confirm that TERT had been removed (data not shown). To our surprise, telomerase activity remained high compared with the control parental culture, which had undetectable activity (Fig. 1a).

Ectopic expression of *c-myc* activates telomerase in HMECs<sup>4</sup>, and hTERT is a direct transcriptional target of *c-myc*<sup>8</sup>. To determine whether activation of *c-myc* was

responsible for the telomerase activity found in our HMEC-Cre cultures, we measured the amount of Myc protein and found a two- to threefold increase in *c-myc* compared with vector control cells (Fig. 1b). This is comparable to the amounts found in a culture immortalized by a *myc*-encoding retrovirus and in a breast-cancer cell line, HBL100 (Fig. 1b). The excision process did not itself cause this increase in *c-myc*, as expression of *c-myc* was high before excision and also in clone 4, which still retained TERT (Fig. 1b).

We examined *c-myc* expression in HMEC-hTERT at different population doublings to determine when it was up-regulated and found that it increased between 107 and 135 population doublings (Fig. 1c). Also, GADD45 protein, whose expression is repressed by Myc, decreased markedly in HMEC-hTERT at PD135 and in HMEC-Cre at PD150 compared with vector control cells and HMEC-hTERT at PD70 (Fig. 1d). These results indicate that,

under standard culture conditions, extension of lifespan by telomerase selects for *c-myc* overexpression in HMECs.

Activation of the *c-myc* oncogene by overexpression, gene amplification, translocation and possibly mutation occurs in a wide variety of tumour types<sup>9</sup>. We have shown that, although telomerase activation extends the lifespan of HMECs, it is also associated with overexpression of *c-myc* and so is not indefinitely genoprotective (even though the chromosome number in such cells is normal; data not shown). Paradoxically, the extension of lifespan that is conferred by TERT causes *c-myc* activation, and this immortalizes cells, in part by activating TERT expression. Furthermore, in HMEC cultures, TERT expression has little, if any, immortalizing potential until the *p16* tumour-suppressor gene has been inactivated<sup>10</sup>. These findings indicate that the use of hTERT for expansion of normal human cells for therapeutic purposes must be approached with caution.

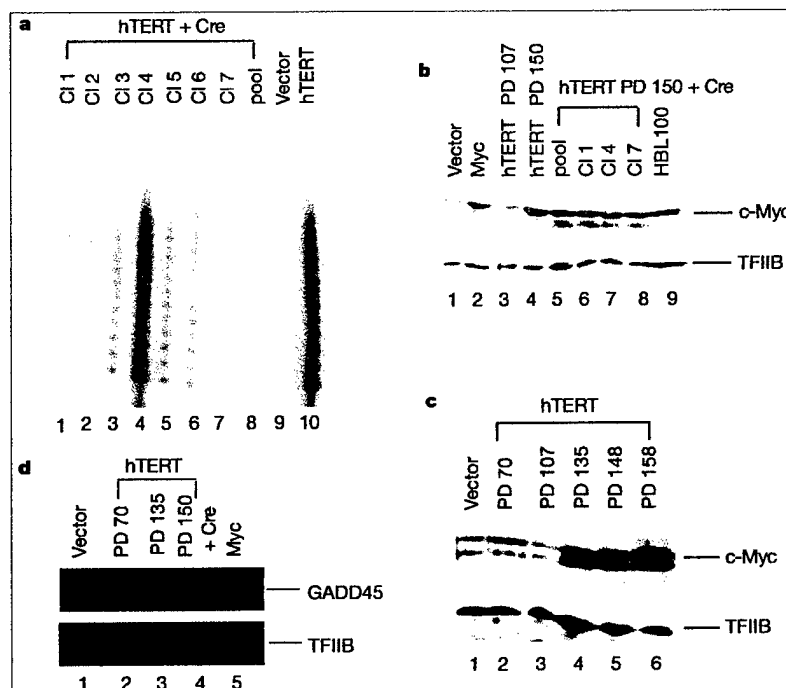


**Figure 1** *c-myc* activity is increased in immortalized HMEC-hTERT cells. HMEC 184 spiral K cells were provided by M. Stampfer. All HMEC-derived cultures were maintained in complete mammary epithelium growth medium (MEGM, Clonetics) under standard conditions<sup>11</sup>. **a**, Telomerase activity in HMEC-Cre cells. We infected HMEC-hTERT cells at PD150 with retroviruses that direct the expression of Cre recombinase, and generated a pool (lane 8) and seven clones (Cl; lanes 1–7). The pool and individual clones, except clone 4, lost the hTERT retroviral cassette. After maintaining cultures for a further 20 population doublings (PDs), we prepared cell lysates for TRAP assays<sup>12</sup>. Lane 9, non-excised HMEC-hTERT cells at PD150. Each lane corresponds to 10,000 cells. Results were similar in clones obtained after a second round of subcloning of Cre clone 6. **b**, Immunoblot using rabbit polyclonal anti-c-Myc antibody (N-262, Santa Cruz) showing c-Myc in cell lysates from vector-infected HMECs (lane 1), cells immortalized by ectopic expression of c-Myc (lane 2), hTERT-expressing cells at PD107 (lane 3) and PD150 (lane 4), Cre-infected HMEC-hTERT pool (lane 5) and clones 1, 4 and 7 (lanes 6–8), and tumour-cell line HBL100 (lane 9). **c**, Immunoblot showing the amount of c-Myc in cell lysates from vector control cells (lane 1) and HMEC-hTERT cells at different PDs (lanes 2–6). **d**, Immunoblot using rabbit polyclonal GADD45 antibody (H-165, Santa Cruz), showing GADD45 in cell lysates from vector control cells (lane 1), hTERT-expressing cells at PD70 (lane 2) and PD135 (lane 3), Cre-infected HMEC-hTERT at PD150 (lane 4) and cells immortalized by ectopic expression of c-Myc (lane 5). In **b–d**, TFIIIB protein was used to normalize loading.

Jing Wang\*, Gregory J. Hannon†, David H. Beach‡

\*Genetica Inc., 1 Kendall Square, Building 600, Cambridge, Massachusetts 02139, USA

†Cold Spring Harbor Laboratory, PO Box 100, Cold Spring Harbor, New York 11743, USA

‡Wolfson Institute for Biomedical Research, University College London, Gower Street, London WC1E 6BT, UK

e-mail: d.beach@ucl.ac.uk

1. Harkley, C. B., Fitcher, A. B. & Greider, C. W. *Nature* 345, 458–460 (1990).
2. Harkley, C. B. *Mutat. Res.* 256, 271–282 (1991).
3. Bodnar, A. G. *et al. Science* 279, 349–352 (1998).
4. Wang, J., Xie, L. Y., Allan, S., Beach, D. & Hannon, G. J. *Genes Dev.* 12, 1769–1774 (1998).
5. Morales, C. P. *et al. Nature Genet.* 21, 115–118 (1999).
6. Jiang, X. R. *et al. Nature Genet.* 21, 111–114 (1999).
7. Sun, P., Dong, P., Hannon, G. J. & Beach, D. *Science* 282, 2270–2272 (1998).
8. Wu, K. J. *et al. Nature Genet.* 21, 220–224 (1999).
9. Alitalo, K. *et al. Biochim. Biophys. Acta* 907, 1–32 (1987).
10. Kiyono, T. *et al. Nature* 396, 84–88 (1998).
11. Foster, S. A. & Galloway, D. A. *Oncogene* 12, 1773–1779 (1996).
12. Kim, N. W. *et al. Science* 266, 2011–2015 (1994).

## Insect perception

### Do cockroaches 'know' about fluid dynamics?

Animals use their senses to extract information from the world around them, so they need to be able to gauge the physical properties of their environment in order to build up an accurate perception of it. For example, a bat needs to 'know' the velocity of sound to estimate how far away an object is, although input to a sensory system may often exploit more complicated properties than this. Here we measure the response by the wind-sensing system of the American cockroach (*Periplaneta americana*) to a complex hydrodynamic flow. We find that the insect's interneurons relay crucial information about the wind's spectral properties, which may warn it of approaching predators.

The cockroach senses minute air movements using tiny hairs on two posterior appendages called cerci<sup>1</sup>. It can surmise the direction of an attack and scurry away to avoid being eaten. Neural signals from the hairs converge on the terminal abdominal ganglion where the wind information is processed, and are then conveyed further by giant interneurons. Although this system has many of the properties of more complex systems<sup>2</sup>, it remains simple enough to be tractable for study.

We produced random wind stimuli with defined spectral properties and measured the average firing rates of several interneurons in response to this stimulus. For a given spectral shape, the total power of the stimulus did not change the steady-state firing rates of the interneurons (Fig. 1a).

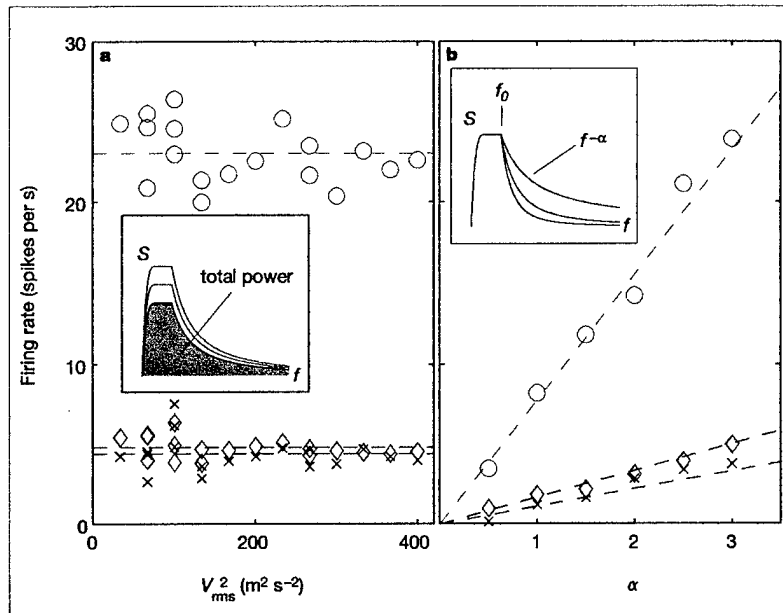


**Figure 1** Average firing rate for random wind stimuli with different spectral parameters. Rates are shown for two typical interneurons (red crosses and blue diamonds) and for all interneurons together (orange circles). Insets show spectral density,  $S$ , as a function of frequency,  $f$ , indicating how the spectral parameters were changed. The frequency  $f_0$  was held constant for these experiments at 10 Hz. The total power, which is directly proportional to the r.m.s. of the square of the wind velocity,  $V_{rms}^2$ , and the high-frequency 'roll-off' parameter,  $\alpha$ , were changed independently. **a**, Firing rate as a function of total power of wind spectra with  $\alpha = 3$ . **b**, Firing rate as a function of  $\alpha$  shows strong dependence on the extent of the high-frequency tail.

Changing the high-frequency roll-off, on the other hand, strongly influenced the firing rates of all of the cells (Fig. 1b). Thus, exposing the system to narrow-band, low-frequency noise produces a strong cell response — that is, a high firing rate — whereas exposure to wide-band stimuli does not. In the limiting case of white noise, the firing rate is almost zero — in spite of the fact that the afferent neurons are known<sup>2</sup> to respond to excitations above 100 Hz. Similar effects are expected for this type of stimulus in other systems<sup>3</sup>.

Let us now consider the typical airflow in a cockroach's environment. The Reynolds number gives an indication of the degree of turbulence<sup>4</sup>: given the typical size of surrounding objects (less than about 1 m in size) and the relevant wind velocities ( $0.1 \text{ m s}^{-1}$ ), the Reynolds number is  $Re \approx 10^3$ , so cockroaches live in a world that is often turbulent. Spectra with long, high-frequency tails are characteristic of turbulent airflow<sup>5</sup>. In contrast, the first sign of an approaching predator is slow-moving air, whose spectrum has only low frequencies: in the case of attacking toads and wasps, timescales are typically about 50 ms — corresponding to frequencies below about 20 Hz (refs 6,7). A low-frequency, narrow-bandwidth stimulus may thus be an indicator of a possible attack.

It is evident from Fig. 1 that the average firing rate of the cockroach interneurons conveys information about the spectral

properties of the prevailing air movement, which change when a predator approaches. Thus, the insect's awareness of these properties and its ability to detect deviations from the norm — in the form of an excess of low-frequency winds — may help it to survive.

**D. Rinberg, H. Davidowitz**

NEC Research Institute, 4 Independence Way, Princeton, New Jersey 08540-6634, USA

e-mail: dima@research.nj.nec.com

1. Camhi, J. M. *Neuroethology* (Sinauer, Sunderland, Massachusetts, 1984).
2. Kondoh, Y., Arima, T., Okuma, J. & Hasegawa, Y. *J. Comp. Physiol. A* 169, 653–662 (1991).
3. Vedenina, V. Y., Rozkova, G. L., Panjutin, A. K., Bykov, A. L. & Kämpfer, G. *J. Comp. Physiol. A* 183, 553–561 (1998).
4. Purcell, E. M. *Am. J. Phys.* 45, 3–11 (1977).
5. Tritton, D. J. *Physical Fluid Dynamics* (Oxford Univ. Press, Oxford, 1998).
6. Plummer, M. R. & Camhi, J. M. *J. Comp. Physiol. A* 142, 347–357 (1981).
7. Gnatzky, W. & Kämpfer, G. *J. Comp. Physiol. A* 167, 551–556 (1990).

## Erratum

### Focusing hard X-rays with old LPs

B. Cederström, R. N. Cahn, M. Danielsson, M. Lundqvist, D. R. Nygren

*Nature* 404, 951 (2000)

An editing error altered the intended meaning of the last two sentences of the seventh paragraph, which should read "We used PVC for focusing. As it contains a large fraction of chlorine, it provides less gain than PMMA, for example." Thus PVC is inferior to PMMA, but we used it for demonstration anyway.

## GREGORY J. HANNON

**EDUCATION:**

|      |                                                |                    |
|------|------------------------------------------------|--------------------|
| 1986 | Case Western Reserve University, Cleveland, OH | B.A., Biochemistry |
| 1991 | Case Western Reserve University, Cleveland, OH | Ph.D. Mol. Biology |

**AWARDS**

|         |                                                              |
|---------|--------------------------------------------------------------|
| 1982-86 | Andrew J. Squire Scholar                                     |
| 1986    | Phi Beta Kappa                                               |
| 1992-95 | Damon Runyon-Walter Winchell Cancer Research Fund Fellowship |
| 1997-00 | Pew Scholar in the Biomedical Sciences                       |
| 2000-03 | Rita Allen Foundation                                        |

**RESEARCH AND PROFESSIONAL EXPERIENCE**

|              |                                                                            |
|--------------|----------------------------------------------------------------------------|
| 2000-present | Associate Professor, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY |
| 1996-2000    | Assistant Professor, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY |
| 1994-1995    | Staff Associate II, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY  |
| 1992-1994    | Postdoctoral Fellow, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY |
| 1986-1992    | Ph.D. Thesis, Case Western Reserve University, Cleveland, OH               |

**ISSUED PATENTS**

|      |                      |                                                          |
|------|----------------------|----------------------------------------------------------|
| 1998 | Patent No. 6,025,192 | Modified Retroviral Vectors                              |
| 1999 | Patent No. 5,962,316 | Cell-Cycle Regulatory Proteins, and Uses Related Thereto |

**PUBLICATIONS:**

Hannon, G.J., Jentcroft, J.E. (1985) MOLECULAR DESIGNER: an interactive program for the display of protein structure on the IBM-PC. *Comput Appl Biosci* 1(3):177-181.

Ransohoff, R.M., Denker, J.A., Takacs, A.M., Hannon, G.J., Nilsen, T.W. (1989) Organization and expression of 5S rRNA genes in the parasitic nematode, *Brugia malayi*. *Nucleic Acids Res.* 17(10):3773-3782.

Maroney, P.A., Hannon, G.J., Nilsen, T.W. (1989) Accurate and efficient RNA polymerase III transcription in a cell-free extract prepared from *Ascaris suum* embryos. *Mol Biochem Parasitol* 35(3): 277-283.

Hannon, G.J., Maroney, P.A., Branch, A., Benenfield, B.J., Robertson, H.D., Nilsen, T.W. (1989) Accurate processing of human pre-rRNA in vitro. *Mol Cell Biol* 9(10):4422-4431.

Maroney, P.A., Hannon, G.J., Nilsen, T.W. (1990) Transcription and cap trimethylation of a nematode spliced leader RNA in a cell-free system. *Proc Natl Acad Sci USA* 87 (2): 709-713.

Hannon, G.J., Maroney, P.A., Ayers, D.G., Shambaugh, J.D., Nilsen, T.W. (1990). Transcription of a nematode trans-spliced leader RNA requires internal elements for both initiation and 3' end-formation. *EMBO J* 9(6):1915-1921.

Hannon, G.J., Maroney, P.A., Denker, J.A., Nilsen, T.W. (1990) Trans splicing of nematode pre-messenger RNA in vitro. *Cell* 60(7): 1247-1255.

Maroney, P.A., Hannon, G.J., Denker, J.A., Nilsen, T.W. (1990) The nematode spliced leader RNA participates in trans-splicing as an Sm snRNP. *EMBO J* 9(11):3667-3673.

- Maroney, P.A., Hannon, G.J., Shambaugh, J.D., Nilsen, T.W. (1991) Intramolecular base pairing between the nematode spliced leader and its 5' splice site is not essential for trans-splicing in vitro. *EMBO J* **10**(12): 3869-3875.
- Hannon, G.J., Chubb, A., Maroney, P.A., Hannon, G., Altman, S., Nilsen, T.W. (1991) Multiple cis-acting elements are required for RNA polymerase III transcription of the gene encoding H1 RNA, the RNA component of human Rnase P. *J Biol Chem* **266**(34): 22796-22799.
- Hannon, G.J., Maroney, P.A., Nilsen, T.W. (1991) U small nuclear ribonucleoprotein requirements for nematode cis- and trans-splicing in vitro. *J Biol Chem* **266**(34): 22792-22795.
- Hannon, G.J., Maroney, P.A., Yu, Y.T., Hannon, G.E., Nilsen, T.W. (1992) Interaction of U6 snRNA with a sequence required for function of the nematode SL RNA in trans-splicing. *Science* **258**(5089): 1775-1780.
- Hannon, G., Demetrick, D. and Beach, D. (1993) Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. *Genes & Dev.* **7**: 2378 -2391.
- Serrano, M. Hannon, G. and Beach, D. (1993) A new regulatory motif in cell cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **336**:704-707.
- Xiong, Y., Hannon, G., Zhang, H., Casso, D., Kobayashi, R. and Beach, D. (1993) p21 is a universal inhibitor of cyclin kinases. *Nature* **336**:701-704.
- Hannon, G.E., Hannon, G.J., Maroney, P.A., Nilsen, T.W. (1994) Transcription of a nematode U1 small nuclear RNA in vitro. 3'-End formation requires cis acting elements within the coding sequence. *J Biol Chem* **269**(17): 12387-12390.
- Zhang, H., Hannon, G.J., Beach, D. (1994) p21-containing cyclin kinases exist in both active and inactive states. *Genes Dev* **8**(15):1750-1758.
- Hannon, G., Casso, D. and Beach, D. (1994) KAP: A dual specificity phosphatase that interacts with cyclin dependent kinases *Proc. Natl. Acad. Sci.* **91**:1731-1735.
- Feilotter, H.E., Hannon, G.J., Rudell, C.J. and Beach, D. (1994) Construction of an improved host strain for two-hybrid screening. *Nucleic Acids Res.* **22**:1502 1503.
- Waga, S., Hannon, G.J., Beach, D. and Stillman, D. (1994) The p21 cyclin dependent kinase inhibitor directly controls ÎNA replication via interaction with PCNA. *Nature* **369**: 574-578.
- Li, R., Waga, S., Hannon, G., Beach, D. and Stillman, B. (1994) Differential effects by the p21 inhibitor on PCNA dependent DNA replication and DNA repair. *Nature* **371**: 534-537.
- Hannon, G. and Beach, D. (1994) p15INK4B is a potential effector of cell cycle arrest mediated by TGF-β. *Nature* **371**:257-261.
- Halevy, O., Novitch, B.G., Spicer, D.B., Skapek, S.X., Rhee, J., Hannon, G.J., Beach, D., Lassar, A.B. (1995) Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267**(5200): 1018-1021.
- Demetrick, D., Matsumoto, S., Hannon, G., Okamoto, K. Xiong, Y., Zhang, H and Beach, D. (1995) Chromosomal mapping of the genes for the human cell cycle proteins cyclin C (CCNC), cyclin E (CCNE), p21 (CDKN1) and KAP (CDKN3). *Cytogenet Cell Gen* **69**: 190-192.
- Okamoto, A., Hussain, S-W., Hagiwara, K., Spillare, E., Rusin, M., Demetrick D., Serrano, M., Hannon, G., Shiseki, M., Zariwala, M., Xiong, Y., Beach, D., Yokota, J., Harris, C. (1995) Mutations in the p16INK4/MTS1/CDKN2, p15INK4B/MTS2 and p18 genes in primary and metastatic lung cancers. *Cancer Research* **55**:1448-1451.
- Macleod, K., Sherry, N., Hannon, G., Beach, D., Kinzler, K., Vogelstein, B. and Jacks, T. (1995) p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes & Dev* **9**:935-944.

- Zhang, H., Hannon, G.J., Casso, D. and Beach, D. (1995) p21 is a component of active cell cycle kinases. *Cold Spring Harbor Symposia on Quantitative Biology*, **LIX**: 21-29.
- Ranade, K., Hussussian, C., Sikorski, R., Varmus, H., Goldstein, A., Tucker, M., Serrano, M., Hannon, G., Beach, D. and Dracopoli, N. (1995) Mutations associated with familial melanoma impair p16INK4 function. *Nature Genetics* **10**:114-116.
- Quelle, D., Ashmun, R., Hannon, G., Rehberger, P., Trono, D., Richter, H., Walker, C., Beach, D., Sherr, C. and Serrano, M. (1995) Cloning and characterization of murine p16INK4a and p15INK4b genes. *Oncogene* **11**:635-645.
- Suzuki, H., Zhou, X., Yin, J., Lei, J., Jiang, H.-Y., Suzuki, Y., Chan, T., Hannon, G., Mergner, W., Abraham, J. and Meltzer, S. (1995) Intragenic mutations of CDKN2B and CDKN2A in primary human esophageal cancers. *Human Molecular Genetics* **4**:1883-1887.
- Orlow, I., Lacombe, L., Hannon, G.J., Serrano, M., Dalbagni, G., Pellicer, I., Reuter, V.E., Zhang, Z.-F., Beach, D. and Cordon-Cardo, C. (1995) Deletion of p16 (INK4A/MTS1) and p15(INK4B/MTS2) in human bladder tumors. *J. Natl. Cancer Inst.* **87**:1524-1529.
- Brugarolas, J., Chandrasekaran, C., Gordon, J., Beach, D., Jacks, T. and Hannon, G. (1995) Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* **377**:552-557.
- Zhou, X., Suzuki, H., Lei, J., Cole, K., Yin, J., Suzuki, Y., Abraham, J.M., Shimada, Y., Imamura, M., Chan, T., Hannon, G.J., Meltzer, S.J. (1996) Growth suppression of esophageal cancer cells by p16(INK4) and p15(INK4B) in vitro. *Cancer J. Sci Am* **2**(4): 221.
- Cobrinik, D., Lee, M.-H., Hannon, G., Mulligan, G., Bronson, R., Dyson, N., Harlow, E., Beach, D., Weinberg, R. and Jacks, T. (1996) Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes & Dev* **10**: 1633-1644.
- Li, R., Hannon, G.J., Beach, D. and Stillman, B. (1996) Subcellular distribution of p21 and PCNA in normal and repair-deficient cells following DNA damage. *Current Biology* **6**: 189-199.
- Terry, L.A., Boyd, J., Alcorta, D., Lyon, T., Solomon, G., Hannon, G., Berchuck, A., Beach, D. and Barrett, J. C. (1996) Mutational analysis of the p21/WAF1/CIP1/SDI1 coding region in human tumor cell lines. *Mol. Carcinogenesis* **16**: 221-228.
- Alcorta, D., Xiong, Y., Phelps, D., Hannon, G., Beach, D. and Barrett, J. Carl (1996) Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proc. Natl. Acad. Sci. USA* **93**: 13742-13747.
- Balbin, M., Hannon, G.J., Pendas, A.M., Ferrando, A.A., Vizoso, F., Fueyo, A. and Lopez-Otin, C. (1996) Functional analysis of a p21WAF1,CIP1,SDI1 mutant (Arg94-->Trp) identified in a human breast carcinoma. Evidence that the mutation impairs the ability of p21 to inhibit cyclin-dependent kinases. *J. Biol Chem* **271** 15782-15786.
- Xie, Q., Sanz-Burgos, A.P., Hannon, G.J. and Gutierrez, C. (1996) Plant cells contain a novel member of the retinoblastoma family of growth regulatory proteins. *EMBO J.* **15**:4900-4908.
- Carnero, A., Hannon, G.J. (1998) The INK4 family of CDK inhibitors. *Curr Top Microbiol Immunol* **227**:43-55.
- Fearnhead, H.O., Rodriguez, J., Govek, E.E., Guo, W., Kobayashi, R., Hannon, G., Lazebnik, Y.A. (1998) Oncogene-dependent apoptosis is mediated by caspase-9. *Proc Natl Acad Sci USA* **95**(23): 13664-13669.
- LaMontagne, K.R., Jr., Hannon, G., Tonks, N.K. (1998) Protein tyrosine phosphatase PTP1B suppresses p210 bcr-abl-induced transformation of rat-1 fibroblasts and promotes differentiation of K562 cells. *Proc Natl Acad Sci USA* **95**(24): 14094-14095.
- Wang, J., Xie, L.-Y., Allan, S., Beach, D. and Hannon, G. (1998) Myc activates telomerase. *Genes & Dev* **12**:1769-1774.

- Sun, P., Dong, P., Dai, K., Hannon, G.J., Beach, D. (1998) p53-Independent role of MDM2 in TGF- $\beta$ 1 resistance. *Science* **282**: 2270-2272.
- 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
- Maestro, R., Dei Tos, A., Hamamori, 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.
- Hudson, J.D., Shoaibi, M., Maestro, R. Carnero, A. Hannon, G. and Beach, D. (1999) A proinflammatory cytokine inhibits p53 tumor suppressor activity. *J. Exp. Med* **190**:1375-1382
- Hammond, S.M., Bernstein, E., Beach, D. and Hannon, G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cell extracts. *Nature* **404**: 293-296.
- Wang, J., Hannon, G.J. and Beach, D. (2000) Risky immortalization by telomerase. *Nature* **405**: 755-756.
- Carnero, A., Hudson, J., Hannon, G. and Beach, D. (2000) Loss-of-function genetics in mammalian cells: the p53 tumor suppressor model. *Nucleic Acid Res* **28**: 2234-2241.