AD_____

Award Number: DAMD17-01-1-0210

TITLE: Genetic Requirements for the Transformation of Human Cells

PRINCIPAL INVESTIGATOR: Yvette R. Seger

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

REPORT DATE: July 2003

TYPE OF REPORT: Annual Summary

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual Summary (1 Jul 2002 - 30 Jun 2003)

4. TITLE AND SUBTITLE
Genetic Requirements for the Transformation of Human Cells

5. FUNDING NUMBERS
DAMD17-01-1-0210

6. AUTHOR(S)
Yvette R. Seger

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

E-Mail: seger@cshl.org

8. PERFORMING ORGANIZATION REPORT NUMBER

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
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Our knowledge of the transformation process has emerged largely from studies of primary rodent cells and animal models. However, numerous attempts to transform human cells using oncogene combinations that are effective in rodents have proven unsuccessful. These findings strongly argue for the study of homologous experimental systems. Here we report that the combined expression of adenovirus E1A, Ha-RasV12, and MDM2 is sufficient to convert a normal human cell into a cancer cell. Notably, transformation did not require telomerase activation. Therefore, activation of telomere maintenance strategies is not an obligate characteristic of tumorigenic human cells.

Activation of telomerase, and consequently telomere maintenance, is a common characteristic of human tumors. Existing models of human cancer cells, created by the introduction of defined genetic alterations, all include telomerase activation as an obligate component of the transformed phenotype. Here we demonstrate that normal human cells can be converted into cancer cells, capable of forming tumors in immunocompromised mice in the absence of telomerase activation or an alternative telomere maintenance strategy. This suggests that alterations in telomere biology must be viewed similarly to genomic instability as catalysts of transformation rather than as central components of the transformed phenotype.

14. SUBJECT TERMS
Suppressors, oncogenes, viruses

15. NUMBER OF PAGES
37

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Much of what we know about the details of neoplastic transformation comes from studies in cell culture. With the original demonstration by Weinberg, Wigler and Barbacid that cells could be transformed *in vitro* by DNA sequences from cancer cells, mouse cells have become premier models for the study of oncogene and tumor suppressor function. This has evolved in recent years to the study of genetically defined mouse models (transgenics and knock-outs). These have provided a great deal of key information regarding the transformation process and have served as model systems in which to test new anti-cancer therapies. Despite the utility of the aforementioned approach, there is a fundamental problem with absolute reliance on this paradigm. Genetic alterations that easily transform rodent cells in culture do not have a similar effect on normal human cells. This implies a difference between the processed of neoplastic transformation in man and mice. In the face intense scrutiny, the nature of this difference has remained a mystery for more than 30 years.

Recent reports from the Weinberg lab (Hahn et al., 1999 and Elenbaas et al., 2001) have begun to illuminate at least one difference between transformation of mouse and human cells—the requirement in human cells for activation of the telomerase enzyme. Our data, however, indicate that this is not the sole key to transformation of all human cell types. Instead, we show that primary human fibroblasts can initially undergo transformation in the absence of telomerase.

This work will provide important information regarding our understanding of the development of breast and other cancers. Chiefly, our work should result in a minimal formula for transforming human diploid fibroblasts and human epithelial cells. It will also provide an opportunity to examine the cellular pathways that must be altered in human cell transformation. However, additional benefits may also accrue. For example, we may be able to create defined human breast cancer cell lines that differ only in single loci (e.g. loss of ARF vs. loss of p53 or loss of Rb vs. loss of p16). These may provide powerful tools for testing drug therapies both *in vitro* and in xenograft models.

Ultimately, our understanding of a cancer cell serves two purposes. First, the study of transformation pathways has, in the past, and will continue to yield important information about how normal cells control their growth. Second, only through understanding the precise molecular events that are required to generate a human tumor may we hope to design rational and specific therapies to battle breast and other cancers.

BODY

The field of cancer biology continues to grow and evolve through the development of new molecular tools and mouse model systems that are used to enhance our understanding of how oncogenes and tumor suppressor work in biological systems to result in the tumor phenotype. Human and rodent cell culture experiments have served as one of the central approaches to determining how these molecular networks interact during tumorigenesis, however one must note that these systems are not always directly comparable. One key example of such a mouse versus human differences is the number of genetic alterations required to convert a normal cell into a cancer cell. While rodent cells can be transformed by two genetic "hits," such as SV40 Large T-antigen or adenoviral E1A in combination with constitutively activated ras (Land et al., 1983; Ruley, 1983), human cells cannot be transformed by these combinations, indicating that the pathways involved in transformation may be different from mouse to human.

A significant and notable difference between rodent and human cells is the requirement for telomerase, the enzyme responsible for maintaining the telomeres during replication. While rodent cells have constitutive telomerase expression in both somatic and germ cells, human cells only have expression in germ cells (reviewed in McEachern et al, 1994). Interestingly, the majority of human tumors are telomerase positive (Kim et al., 1994), indicating a role for telomerase activation in tumorigenesis. In a series of publications, Weinberg and colleagues have shown that primary human fibroblasts, mammary epithelial, and small airway epithelial cells can be transformed by coexpression of the SV40 early region (large T-antigen and small t-antigen), the catalytic subunit of telomerase, hTERT, and constitutively activated Harvey Ras (V12) (Hahn et al, 1999; Elenbaas et al., 2001; Hahn et al. 2002; Lundberg et al., 2002).

While the majority of human cancers indicate increased ras expression (typically through an activating mutation), recapitulation of this event has proven difficult in primary cell culture systems, as ras overexpression initiates a severe and irreversible growth arrest program termed senescence (Serrano et al., 1997). While several proteins have been shown to bypass ras-induced senescence in primary mouse embryonic fibroblasts (i.e. E1A, Bmi-1, Tbx-2, DRIL1) (Serrano et al, 1997, Jacobs et al., 1999, Jacobs et al., 2001, Peeper et al., 2002), only E1A has been shown to effectively bypass this effect in primary human fibroblasts (Serrano et al., 1997). It is also interesting to note that primary human skin fibroblasts (BJ, WI-38, DET 551, and HS 68) co-expressing E1A and Ha-rasV12 are capable of *in vitro* transformation in an assay for anchorage-independent growth (Seger et al., 2002.) These E1A + Ras expressing cells, however, do not result in tumorigenesis in immunocompromised mice, and require a third genetic alteration, MDM2 overexpression, to result in *in vivo* transformation (Seger et al., 2002).

In the first-year report for this project, results pertaining to the characterization of the E1A + MDM2 + Ha-RasV12 human cell transformation model (Task #1) were outlined. These results culminated in a peer-reviewed publication in November 2002. Currently, experimentation has focused upon the functional interactions required for E1A-mediated transformation. We are taking a two-pronged approach to this problem. First, I am testing a small subset of genes for the ability to complement and rescue

transformation defects of specific E1A functional mutants. The second approach is to screen a library of genes represented by short-hairpin RNA constructs for gene silencing capable of complementing an E1A functional mutant. Finally, I am continuing to test logically chosen combinations of cellular oncogenes for the development of an entirely cellular transformation model.

TASK #1: MODELS FOR HUMAN CELL TRANSFORMATION

As was indicated in the first-year (2002) report, the goals of this task are now complete and have resulted in a publication, 'Transformation of normal human cells in the absence of telomerase activation' (Cancer Cell 2: 401-413 [2002]). The reprint of this publication is included as an appendix to this report.

TASK #2: GENETIC REQUIREMENTS FOR HUMAN CELL TRANSFORMATION MEDIATED BY ADENOVIRUS E1A

The work on this particular task has been the focus of my experiments since the 2002 report. Thus far, the majority of the experiments have utilized the classical oncogene complementation approach as outlined in the original fellowship proposal. Our main focus has been on oncogene complementation of two fairly well-characterized regions of E1A: the 26-35 region at the N-terminus and the highly conserved Rb binding motif, CR2.

The 26-35 region has been shown to be critical for E1A to interact with the p400 and TRRAP transcriptional coactivators. Interestingly enough, these coactivators were originally discovered through their interactions with the c-Myc oncogene. Therefore I tested the ability of c-Myc to rescue the transformation defect of fibroblasts coexpressing E1AΔ26-35 + Ha-RasV12. While we currently have positive *in vitro* data in hand, I am waiting for results from *in vivo* tumorigenicity assays. I predict that we will have a more complete *in vivo* picture in approximately 6 months after several rounds of injections into immunocompromised mice.

The CR2 region is responsible for binding the Rb tumor suppressor, the phosphorylation status of which regulates E2F-mediated transcription, and thus progression of the cell cycle to S phase. The complex of cyclin E and CDK2 is responsible for this hyper-phosphorylation of Rb. Apoptosis studies have indicated that E1AΔCR2 could be functionally complemented by cyclin E expression (A. Samuelson, personal communication.) We therefore tested the ability of cyclin E expression to rescue the E1AΔCR2 mutation in transformation. As is indicated in the results section, E1AΔCR2 + Cyclin E + Ha-RasV12 expressing cells are capable of relatively normal proliferation, indicating that cyclin E is in fact competent to rescue the proliferative defect of E1AΔCR2 + Ha-RasV12 cells. While these cells are capable of proliferation, they are unable to grow in an anchorage-independent manner as tested by growth in soft agar. This result indicates that while cyclin E expression may be sufficient to rescue apoptotic pathways in cells expressing E1AΔCR2, it is not the correct complement for the transformation defects of the E1AΔCR2 mutant.

In addition to doing classical oncogene complemention assays, my project is now beginning to benefit from the studies of RNA interference (RNAi) mechanisms going on

in the rest of the Hannon lab through the use of short hairpin RNA-expressing (shRNA) retroviral vectors. In order to determine how well these shRNA retroviruses would work in my transformation system, I tested the ability of a Rb hairpin construct to rescue proliferation and/or transformation defects of cells expressing E1AΔCR2 + Ha-RasV12. As was outlined in the results section, E1AΔCR2 + Rb hairpin + Ha-RasV12 expressing cells were not only capable of proliferation, they also exhibited morphological hallmarks of transformed cells, such as spindle morphology and focus formation.

These results are extremely preliminary, and I am continuing to repeat the experiments using an expanded panel of Rb hairpins from Masashi Narita and Jack Zilfou (Lowe lab). These cells have been plated in soft agar to test for anchorage-independent growth and have also been injected into immunocompromised mice to test for tumor formation. As with the c-myc complementation experiments, I predict a more complete set of results within the time period of six to eight months.

A. Determination of the functional interactions of E1A required for the transformation of human cells

c-Myc is able to complement E1AΔ26-35 to result in in vitro transformation

The amino terminus of E1A is known to bind a variety of transcriptional coactivators such as p300, CBP, and the p400 complex (Dorsman et al, 1995; Wang et al., 1995; Goodman and Smolik, 2000; McMahon et al., 1998; Barbeau et al., 1994). In order to determine which of these interactions were essential for E1A-mediated transformation, we tested a series of well-characterized E1A functional mutants for their ability to cooperate with MDM2 and Ha-rasV12 in *in vitro* transformation. Much to our surprise, none of these mutants were capable of significant cooperativity (Appendix Figure 1). One particular mutation, Δ26-35, was capable of weak colony formation in soft agar when coexpressed with MDM2 and Ha-RasV12 (Appendix Figure 2).

The E1AΔ26-35 mutant is of particular interest as the deleted region has been shown to bind the p400 coactivator complex and the myc-interacting protein, TRRAP. In rodent cells, this region has been shown to be essential for transformation mediated by E1A (Fuchs et al., 2001). In BJ human fibroblasts, co-expression of E1AΔ26-35 and Ha-RasV12 results in a cell population that is viable and proliferative, yet incapable of anchorage-independent growth in soft agar. Since both p400 and TRRAP have been shown to interact with the c-myc oncoprotein, we were curious to test whether co-expression of E1AΔ26-35 + c-myc + Ha-RasV12 would result in anchorage independent growth. In soft agar assays, BJ fibroblasts triple-infected with E1AΔ26-35 + c-myc + Ha-rasV12 were capable of robust colony formation in soft agar. Colony size was comparable to the colonies of E1A + MDM2 + Ha-RasV12 expressing cells, whereas E1AΔ26-35 + Ha-RasV12 expressing cells resulted in no colony formation (Appendix Figure 2).

Cyclin E expression is capable of complementing E1AΔCR2 to rescue proliferation, however it is incapable of rescuing transformation

E1A is able to modulate the activity of the E2F family of transcription factors, and thus entry into S phase, by binding members of the Rb family through conserved regions designated CR1 and CR2 (Whyte et al., 1988; Harlow et al., 1986; Whyte et al.,

1989). The E1AΔCR2 mutant is completely defective for pRb binding, and BJ fibroblasts co-expressing E1AΔCR2 and Ha-RasV12 are senescent. In his experiments testing complementation of E1A mutants to re-establish apoptosis, Andy Samuelson (Lowe Lab, CSHL), determined that Cyclin E expression was sufficient to rescue the apoptotic defects of fibroblasts expressing the E1AΔCR2 mutant. In my transformation-based system, while I found that Cyclin E expression was, in fact, sufficient to rescue the proliferative defects of fibroblasts coexpressing E1AΔCR2 + Ha-RasV12, these cells were unable to result in anchorage-independent growth in soft agar (Appendix Figure 3).

Co-expression of c-Myc and cyclin E is insufficient to bypass ras-induced senescence

With the results that c-Myc could successfully complement an N-terminal deletion of E1A in an *in vitro* transformation assay and that cyclin E could rescue the proliferation defect conferred by the deletion of the CR2 region, we hypothesized that full-length E1A could be complemented in the E1A + Ras transformation equation by co-expression of cyclin E and c-Myc. This hypothesis was also based upon results in apoptosis studies where c-myc and cyclin E expression could rescue drug-induced apoptosis in IMR90 human fibroblasts (A. Samuelson, personal communication).

BJ fibroblasts were triple-infected with c-Myc + human cyclin E + Ha-rasV12, as had been previously done in the original E1A + MDM2 + Ha-RasV12 experiments. Much to our surprise, the majority of the resultant cell population was severely senescent. Apparently, one caveat to translating the apoptosis results to our transformation model was that the apoptosis experiments did not require use of Ha-RasV12, therefore we were unaware that the combination of c-Myc and cyclin E would be incapable of bypassing ras-induced senescence (Appendix Figure 4).

E1AΔCR2 can be rescued by a short-hairpin RNA (shRNA) that targets pRb

In addition to examining which genes can be overexpressed in combination to result in the tumorigenic phenotype, we have also begun a series of experiments in which targeted gene silencing is used to rescue the transforming capabilities of E1A mutants. At the time of the 2002 report, the technology did not yet exist to transduce shRNAs from retroviral constructs. This technology is now available and in widespread use at CSHL.

The first E1A target we have attempted to silence has been pRb, utilizing hairpins designed by Masashi Narita and Jack Zilfou (Lowe Lab, CSHL.) The first experiment was to test the ability of the Rb hairpin to complement the E1AΔCR2 to rescue transformation. BJ fibroblasts were triple infected with E1AΔCR2 + Rb Hairpin (MN#2) + Ha-RasV12 and drug selected as in other experiments. The majority of these cells were capable of proliferation, in contrast to fibroblasts expressing only E1AΔCR2 + Ha-Rasv12, which are senescent (Appendix Figures 5, 6, 7). Interestingly, the majority of these cells not only regained proliferative capabilities, but also exhibited traits of transformed cells, namely spindle morphology and lack of contact inhibition to result in visible focus formation. While these results are still extremely preliminary, it does offer some proof of principle in terms of using shRNAs to evaluate the role of E1A targets in transformation.

B. Determination of the role of MDM2 in E1A-mediated human cell transformation

As part of the 2002 update, we presented information indicating potential revisions to this specific task based upon *in vivo* data. In brief, MDM2 was unable to completely rescue the transformation defects of E1A mutants with N-terminal deletions. We had also tested anti-apoptotic genes, namely a dominant-negative p53 (175H) and bcl-2, for the ability to substitute for MDM2 in transformation. Both dn-p53 and bcl-2 are capable of cooperating with E1A + Ras to promote anchorage-independent growth in soft agar (Appendix Figure 8), however, only cells co-expressing E1A + dn-p53 + Ha-RasV12 were capable of tumor formation in nude mice. This result indicated that the major function of MDM2 in E1A-mediated transformation was disruption of the p53 pathway.

In light of these results, the majority of this task is complete. Since these aforementioned results indicate the importance of p53 pathway disruption in E1A-mediated tumorigenesis, we would also like to test the ability p53 shRNAs to promote tumorigenesis in E1A + Ras expressing cells. After assembling a series of human p53 shRNAs similar to those described for murine p53 (Hemann et al., 2003), we can compare E1A + MDM2 + Ha-Ras tumors to E1A + p53 shRNA + Ha-Ras for potential differences in tumor formation rates and comparative tumor volume.

KEY RESEARCH ACCOMPLISHMENTS

TASK #1: Creation of Human Tumor Cell Models

- Complete and published, November 2002

TASK #2; Genetic Requirements for Human Cell Transformation

- Functional mutants of E1A indicate that binding of pRB, p300, and p400/TRRAP are essential for the ability of E1A to promote transformation.
- Use of a functional E1A mutant unable to bind CtBP indicates that this interaction is not required for the ability of E1A to promote transformation.
- c-Myc is able to complement E1AΔ26-35 to result in *in vitro* transformation indicating p400/TRRAP binding contributes to the transformed phenotype.
- Cyclin E expression is capable of complementing E1AΔCR2 to rescue proliferation, however it is incapable of rescuing transformation, indicating key differences between apoptotic and transformation pathways mediated by E1A.
- Co-expression of c-Myc and cyclin E is insufficient to bypass ras-induced senescence, indicating E1A functions in addition to p400/TRRAP and pRB pathways are required for transformation.
- E1AΔCR2 can be rescued by a short-hairpin RNA (shRNA) that targets pRb, and the morphology of cells coexpressing E1AΔCR2 + shRNA-Rb + Ha-RasV12 or E1AΔCR2 + shRNA-Rb + MDM2 + Ha-RasV12 indicates not only rescue of the CR2 deletion for proliferation, but also for transformation *in vitro*.

REPORTABLE OUTCOMES

Manuscripts

Seger, Y.R., Garcia-Cao, M., Piccinin, S., Lo Cunsolo, C., Doglioni, C., Blasco, M.A., Hannon, G.J., and Maestro, R. (2002). Transformation of normal human cells in the absence of telomerase activation. Cancer Cell *2:* 401-413.

Abstracts/Posters

2000

Seger, Y.R., Sun, P., and Hannon, G.J. "Genetic Requirements for the Transformation of Human Cells." DOD-Era of Hope Breast Cancer Meeting, Atlanta, GA, USA

Seger, Y.R., Sun, P., and Hannon, G.J. "Genetic Requirements for the Transformation of Human Cells." Cancer Genetics & Tumor Suppressor Genes Meeting, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA

2001

Seger, Y.R., Maestro, R., Sun, P., and Hannon, G.J. "Genetic Requirements for the Transformation of Normal Human Cells." Telomeres & Telomerase Meeting, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA

Seger, Y.R., Maestro, R., Sun, P., and Hannon, G.J. "Transformation of Human Cells in the Absence of Telomerase Activation." AACR National Meeting, New Orleans, LA, USA

2002

Seger, Y.R., Maestro, R., Piccinin, S., Garcia-Cao, M., Blasco, M.A., and Hannon, G.J. "Transformation of Human Cells in the Absence of Telomerase Activation." Keystone Symposium on the Genomics and Genetics of Senescence and Cancer, Keystone, CO, USA

Seger, Y.R., Garcia-Cao, M., Piccinin, S., Lo Cunsolo, C., Doglioni, C., Blasco, M.A., Hannon, G.J., and Maestro, R. "Transformation of normal human cells in the absence of telomerase activation." Cancer Genetics & Tumor Suppressor Genes Meeting, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA

Seger, Y.R., Garcia-Cao, M., Piccinin, S., Lo Cunsolo, C., Doglioni, C., Blasco, M.A., Hannon, G.J., and Maestro, R. "Transformation of normal human cells in the absence of telomerase activation." DOD-Era of Hope Breast Cancer Meeting, Orlando, FL, USA

Presentations

Seger, Y.R., Maestro, R., Piccinin, S., Garcia-Cao, M., Blasco, M.A., and Hannon, G.J. "Transformation of Human Cells in the Absence of Telomerase Activation." Keystone Symposium on the Genomics and Genetics of Senescence and Cancer, Keystone, CO, USA

CONCLUSION

In conclusion, the work that has been completed since the 2002 report has focused upon determining the functional interactions required by E1A to promote tumorigenesis in the E1A + MDM2 + Ras model. The development of shRNA retroviruses and the assembly of the shRNA library have streamlined many of the laborious mutant complementation experiments that were an initial part of this proposal. Use of such technology will assist us in our ultimate goal of determining the minimum pathway requirements in E1A/MDM2/RasV12-mediated tumorigenesis.

REFERENCES

Barbeau, D., Charbonneau, R., Whalen, S.G., Bayley, S.T., and Branton, P.E. (1994). Functional interactions within adenovirus E1A protein complexes. Oncogene. *9*, 359-373.

Dorsman, J.C., Hagmeyer, B.M., Veenstra, J., Elfferich, N.N., Zanntema, A., and Van Der Ebb, A.J. (1995). The N-terminal reion of the adenviirus type 5 E1A proteins can repress expression of cellular genes via two distinct but overlapping domains. J. Virol., *69*, 2962-2967.

Elenbaas, B., Spirio, L., Koerner, F., Fleming, M.D., Zimonjic, D.B., Donaher, J.L., Popescu, N.C., Hahn, W.C., and Weinberg, R.A. (2001). Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev. *15*, 50-65.

Fuchs, M., Gerber, J., Drapkin, R., Sif, S., Ikura, T., Ogryzko, V., Lane, W.S., Nakatani, Y., and Livingston, D.M. (2001). The p400 complex is an essential E1A transformation target. Cell. *106*, 297-307.

Goodman, R.H. and Smolik, S. (2000). CBP/p300 in cell growth, transformation, and development. Genes Dev. *14*, 1553-1577.

Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, R.A. (1999). Creation of human tumor cells with defined genetic elements. Nature. *400*, 464-468.

Hahn, W.C., Dessain, S.K., Brooks, M.W., King, J.E., Elenbaas, B., Sabatini, D.M., DeCaprio, J.A., and Weinberg, R.A. (2002). Enumeration of the simian virus 40 early region elements necessary for human cell transformation. Mol Cell Bio. 22, 2111-23.

Harlow, E., Whyte, P., Franza, B.R., Jr., and Schley, C. (1986). Association of adenovirus early-region 1A proteins with cellular polypeptides. Mol Cell Biol. *6*, 1579-1589.

Hemann, M.T., Fridman, J.S., Zilfou, J.T., Hernando, E., Paddison, P.J., Cordon-Cardo, C., Hannon, G.J., and Lowe, S.W. (2003). An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes in vivo. Nat Genet. *33*, 396-400.

Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. (1999). The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature. *397*, 164-168.

Jacobs, J.J., Keblusek, P., Robanus-Maandag, E., Kristel, P., Lingbeek, M., Nederlof, P.M., van Welsem, T., van de Vijver, M.J., Koh, E.Y., Daley, G.Q., and van Lohuizen,

M. (2000). Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19(ARF)) and is amplified in a subset of human breast cancers. Nat Genet. *26*, 291-299.

Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L., Coviello, G.M., Wright, W.E., Weinrich, S.L., and Shay, J.W. (1994). Specific association of human telomerase activity with immortal cells and cancer. Science. 266, 2011-2015.

Land, H., Parada, L.F., and Weinberg, R.A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature. *304*, 596-602.

Lundberg, A.S., Randell, S.H., Stewart, S.A., Elenbaas, B., Hartwell, K.A., Brooks, M.W., Fleming, M.D., Olsen, J.C., Miller, S.W., Weinberg, R.A., and Hahn, W.C. (2002). Immortalization and transformation of primary human airway epithelial cells by gene transfer. Oncogene. *21*, 4577-4586.

McEachern, M.J., Krauskopf, A., and Blackburn, E.H. (2000). Telomeres and their control. Ann Rev Genet. *34*, 331-358.

McMahon, S.B., Van Buskirk, H.A., Dugan, K.A., Copeland, T.D., and Cole, M.D. (1998). The novel ATM-related protein TRRAP is an essential coactivator for the c-myc and E2F oncoproteins. Cell. *94*, 363-374.

Paddison, P.J., Caudy, A.A., Bernstein, E. Hannon, G.J. and Conklin, D.S. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes & Dev. *16*, 948-958.

Peeper, D.S., Shvarts, A., Brummelkamp, T., Douma, S., Koh, E.Y., Daley, G.Q., and Bernards, R. (2002). A functional screen identifies hDRIL1 as an oncogene that rescues RAS-induced senescence. Nat Cell Biol. *4*,148-153.

Ruley, H.E. (1983). Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature. *304*, 602-606.

Seger, Y.R., García-Cao, M., Piccinin, S., Lo Cunsolo, C., Doglioni, C., Blasco, M., Hannon, G.J., and Maestro, R. (2002). Transformation of normal human cells in the absence of telomerase activation. Cancer Cell. *2*, 401-413.

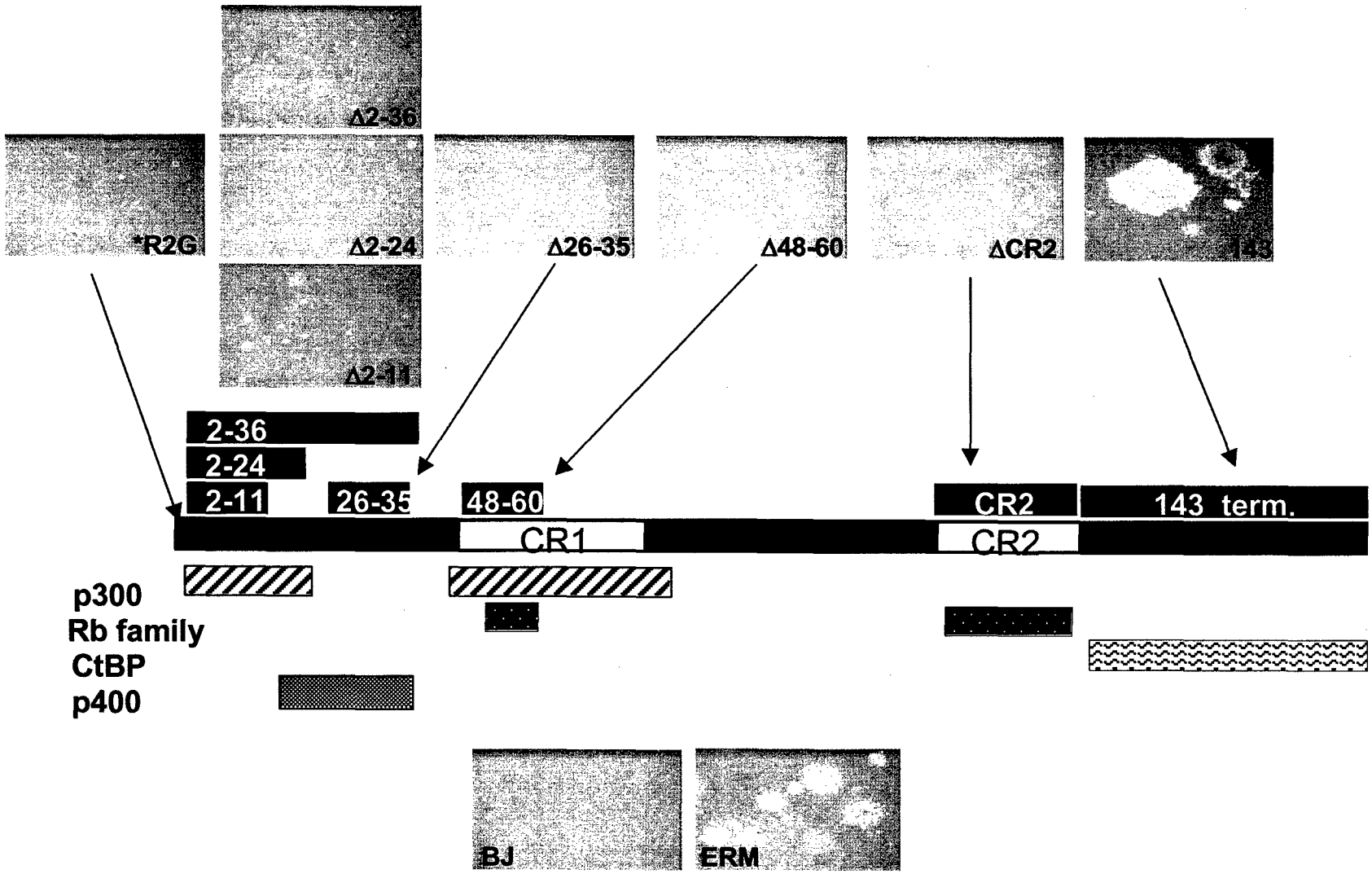
Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D.H., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell. *88*, 593-602.

Wang, H.H., Moran, E., annd Yaciuk, P. (1995). E1A promotes association between p300 and Rb in multimeric complexes required for normal biological activity. J. Virol. *69*, 7917-7924.

Whyte, P., Buchkovich, K.J., Horowitz, J.M., Friend, S.H., Raybuck, M., Weinberg, R.A., and Harlow, E. (1988). Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature. *334*, 124-129.

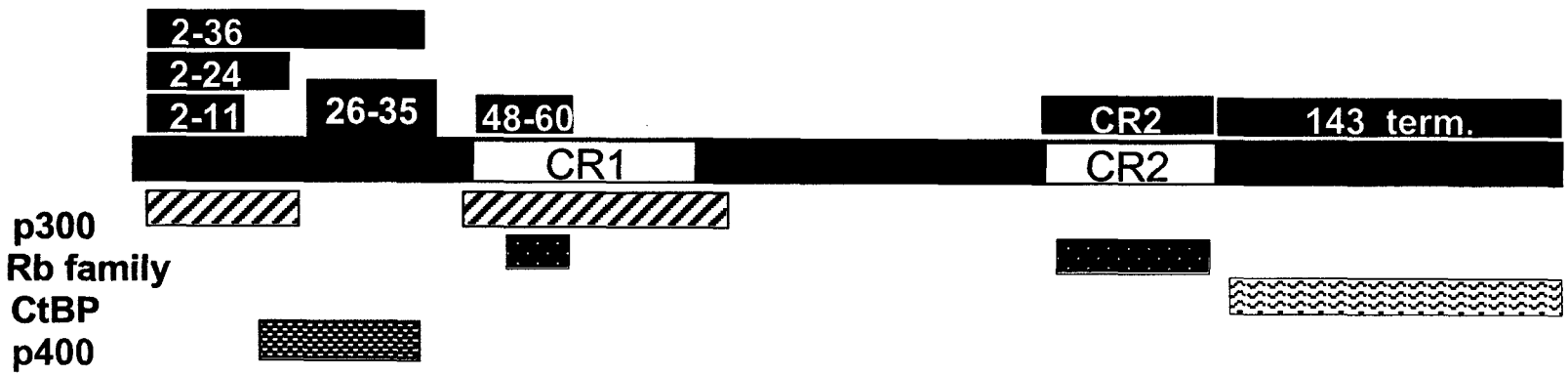
Whyte, P., Williamson, N.M., and Harlow, E. (1989). Cellular targets for transformation by the adenovirus E1A proteins. Cell. *56*, 67-75.

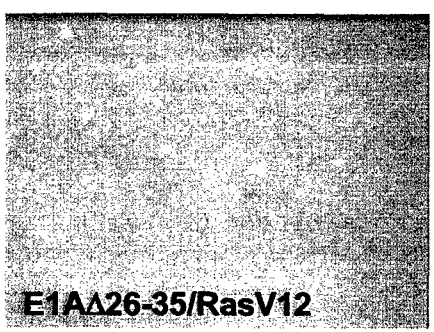
E1A functional mutants reveal essential interactions for E1A/Ras-mediated transformation

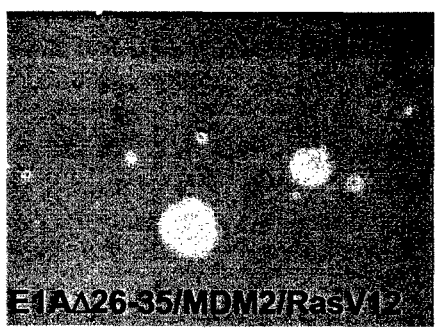


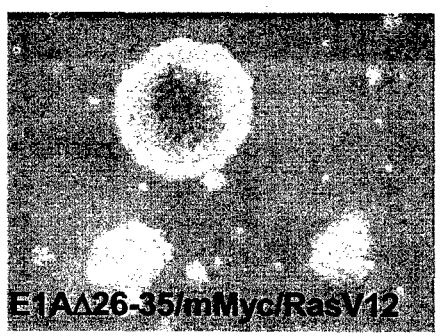
APPENDIX FIGURE 1

Rescue of *in vitro* transformation defect of E1AΔ26-35 by complementation with c-Myc



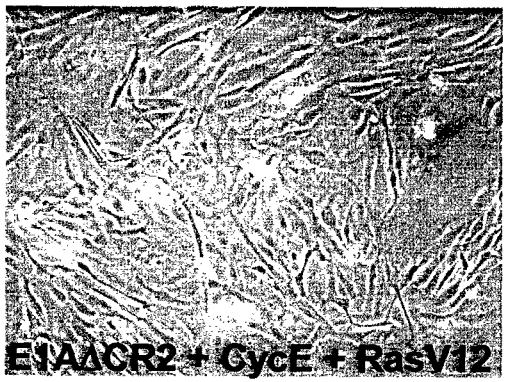


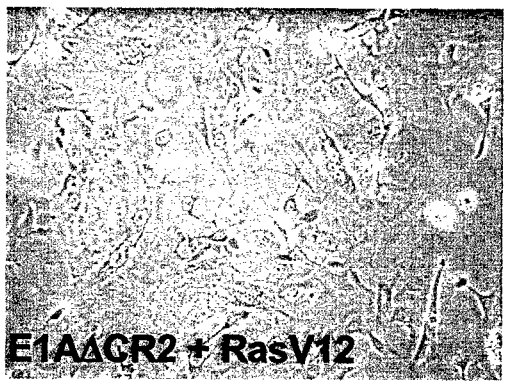




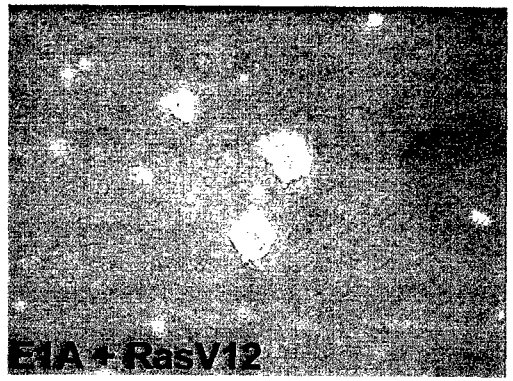
APPENDIX FIGURE 2

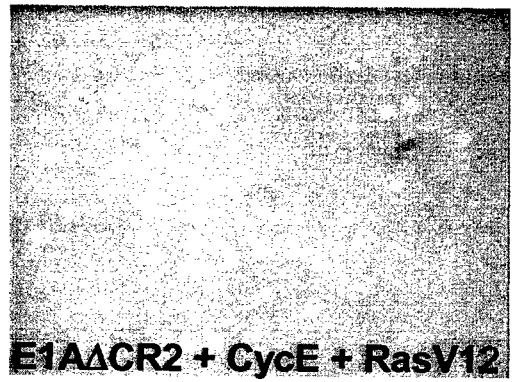
Cyclin E is unable to complement E1AΔCR2 to rescue transformation

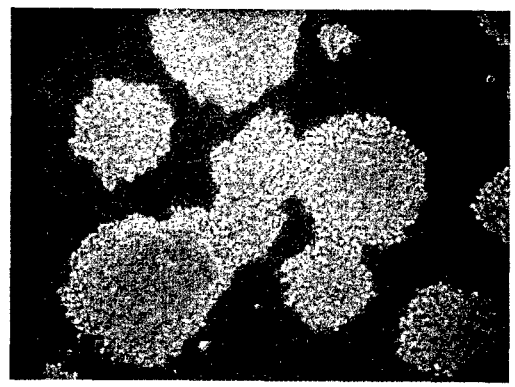


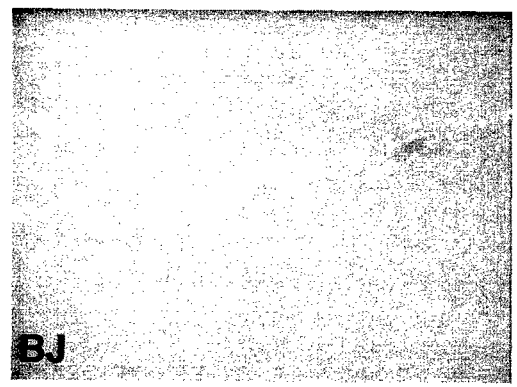


Cultured Cells





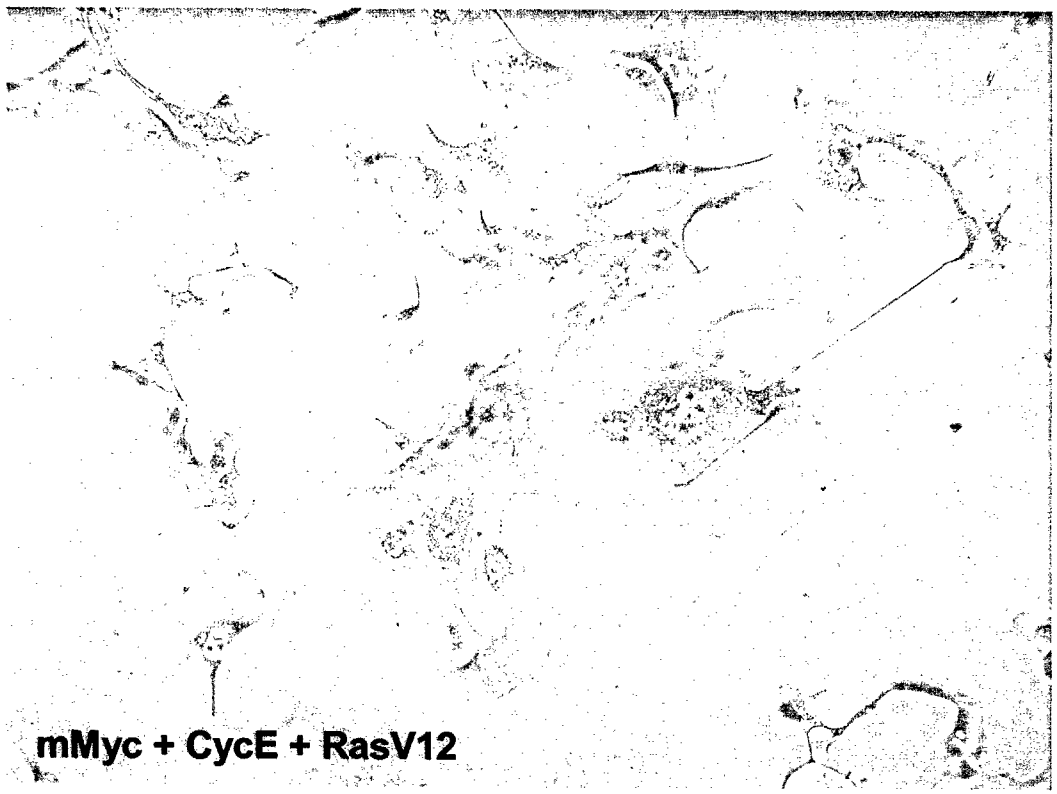




Day 10 Soft Agar

APPENDIX FIGURE 3

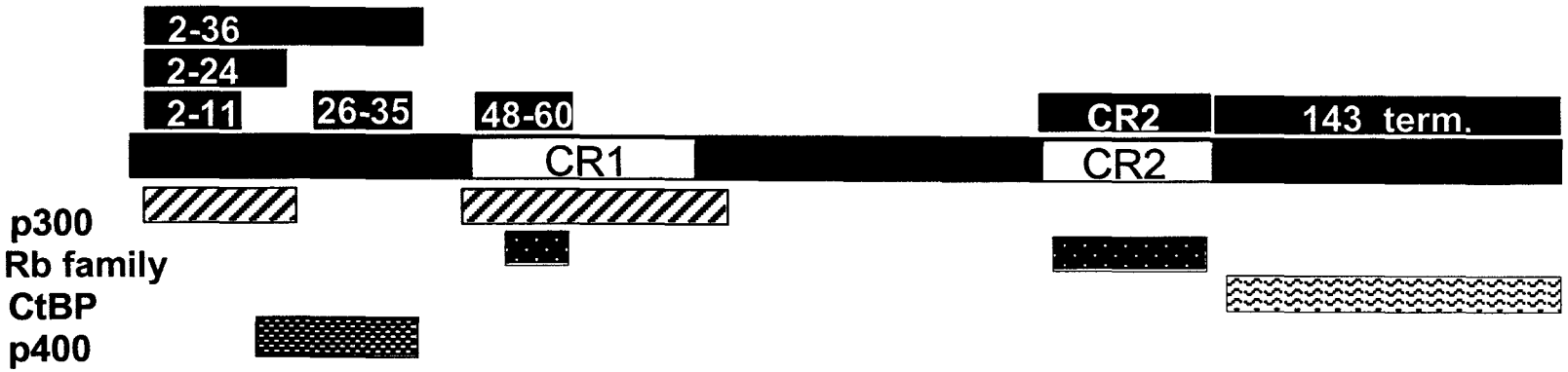
Coexpression of myc and cyclin E is insufficient to bypass ras-induced senescence

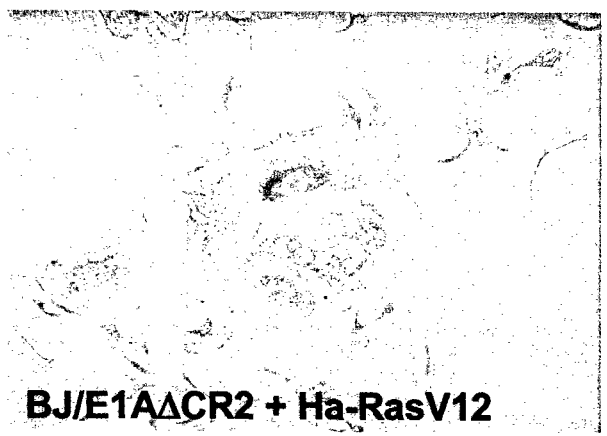


Staining for the senescence-associated β-galactosidase marker

APPENDIX FIGURE 4

Use of a Rb-shRNA to rescue the proliferation and/or transformation defects of the E1AΔCR2 mutant

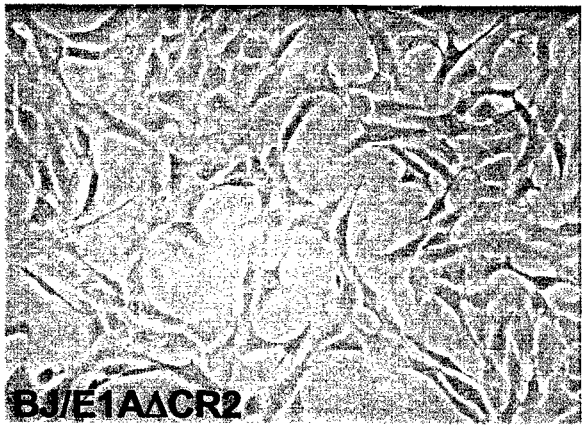




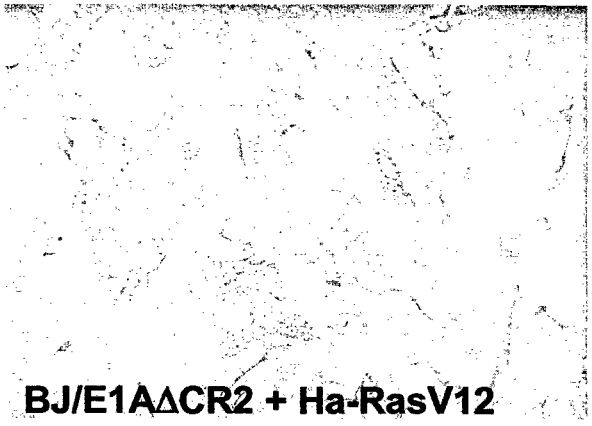
Staining for the senescence-associated β-galactosidase marker

APPENDIX FIGURE 5

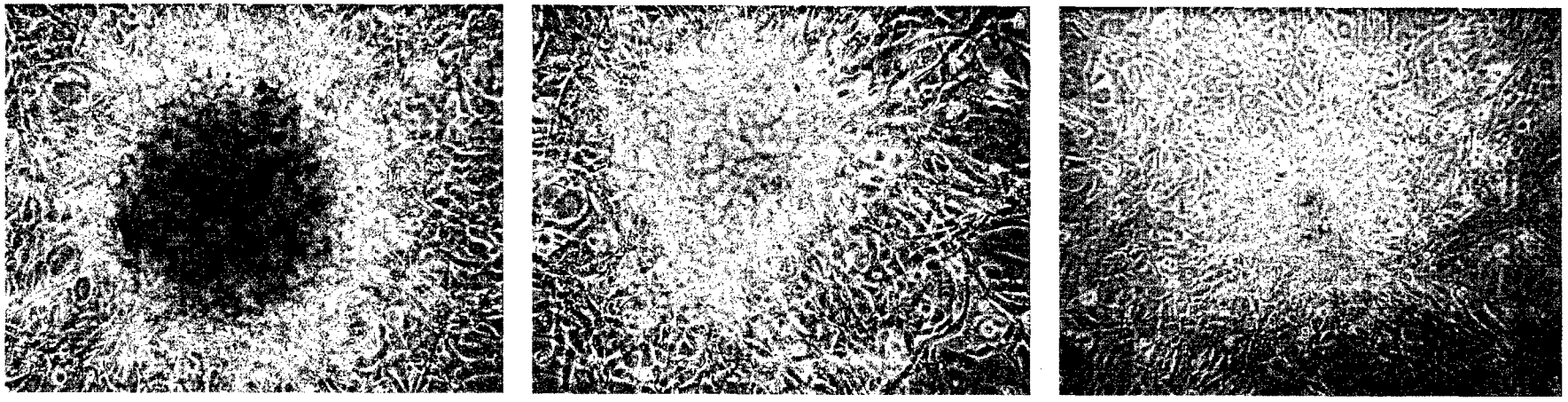
Coexpression of E1AΔCR2 + Rb shRNA + RasV12 results in focus formation in culture



BJ/E1AΔCR2



BJ/E1AΔCR2 + Ha-RasV12

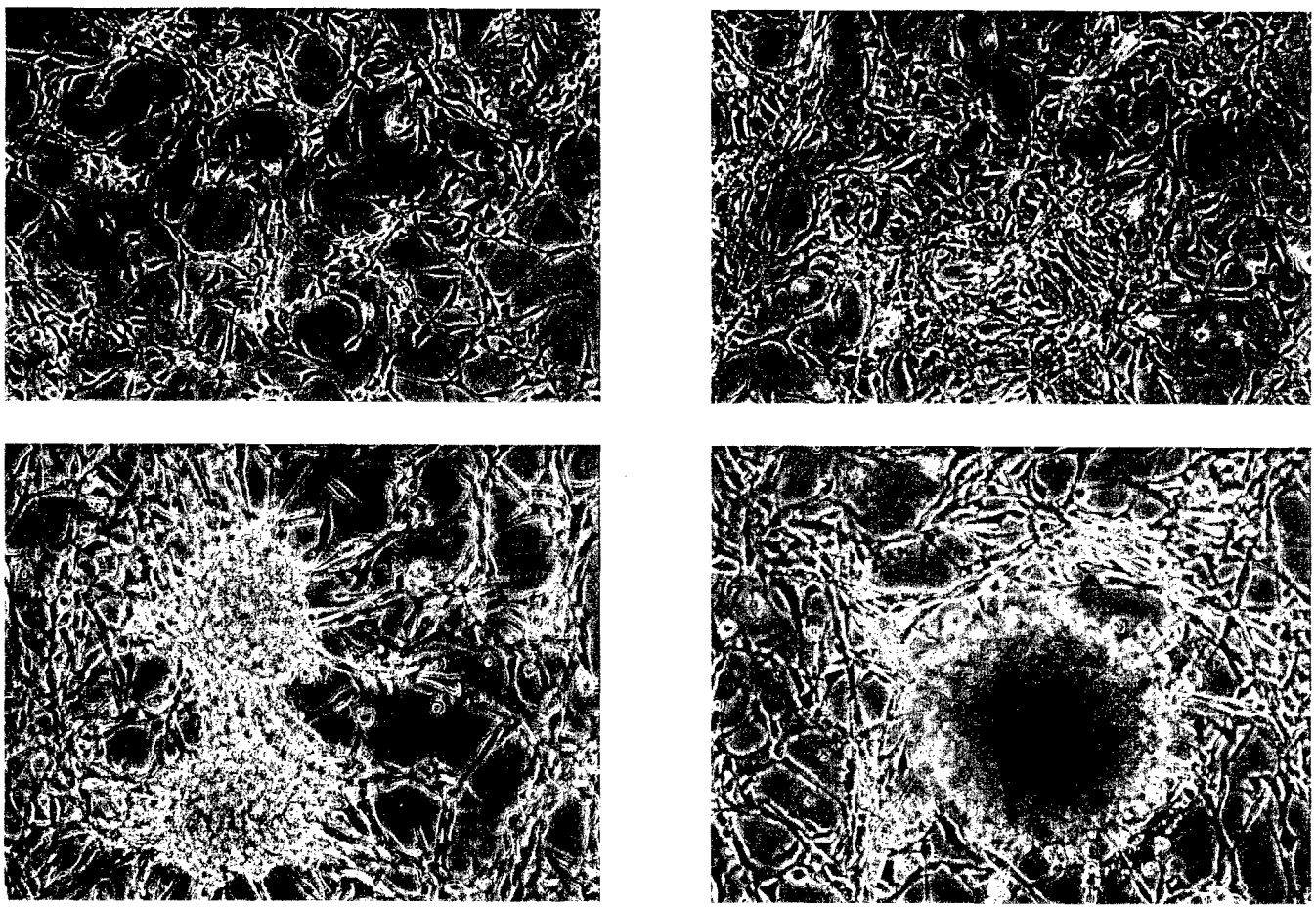


BJ/E1AΔCR2 + Rb-Hairpin + Ha-RasV12

APPENDIX FIGURE 6

Quadruple Infections

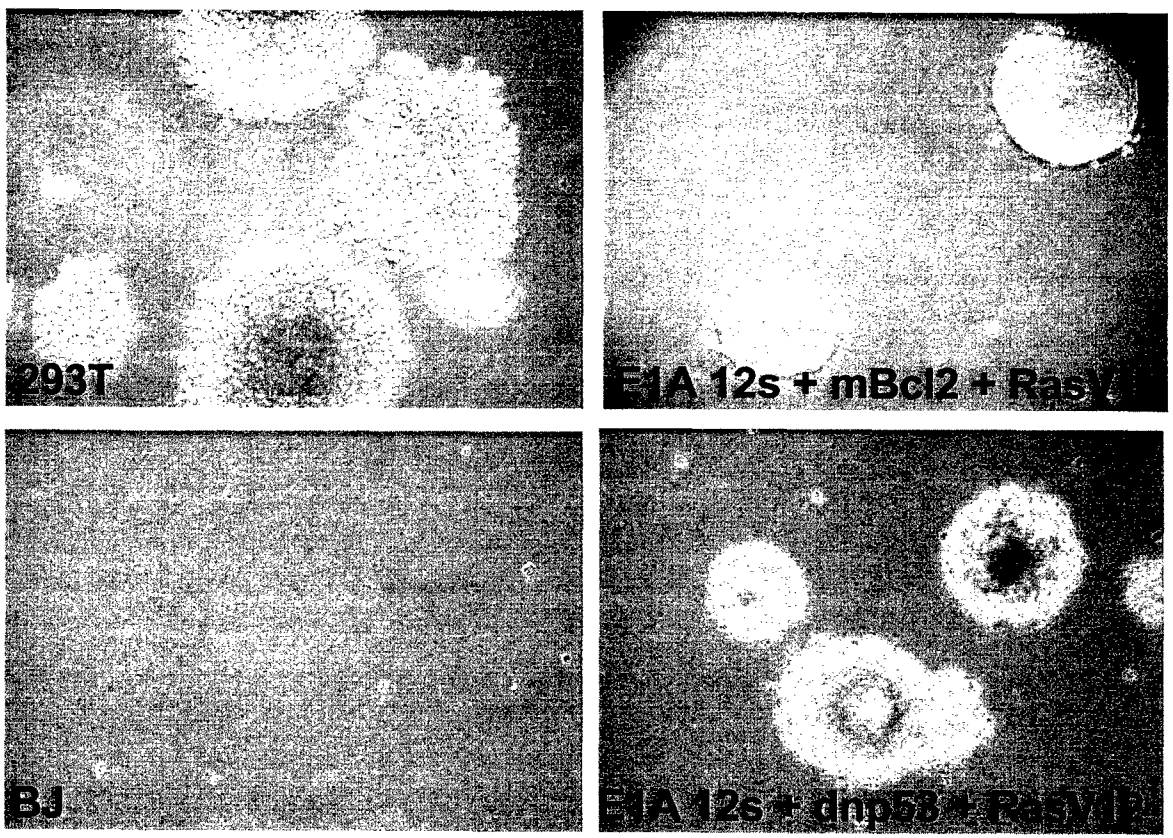
E1AΔCR2 + RbHP2 + MDM2 + Ha-RasV12



Puromycin vector
Neomycin vector
Hygromycin vector

APPENDIX FIGURE 7

Both dn-p53(175H) and mBcl-2 are capable of cooperating with E1A + RasV12 to promote colony formation in soft agar



APPENDIX FIGURE 8

Transformation of normal human cells in the absence of telomerase activation

Yvette R. Seger,[1,2] Marta García-Cao,[3] Sara Piccinin,[4] Crocifissa Lo Cunsolo,[5] Claudio Doglioni,[5] María A. Blasco,[4] Gregory J. Hannon,[1,6] and Roberta Maestro[4]

[1]Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, New York 11724
[2]Graduate Program in Genetics, State University of New York at Stony Brook, Stony Brook, New York 11794
[3]Department of Immunology and Oncology, Centro Nacional de Biotecnología-CSIC, Campus Cantoblanco, 28049 Madrid, Spain
[4]MMNP Unit—Experimental Oncology, CRO-IRCCS, National Cancer Institute, Via Pedemontana Occ. 12, 33081 Aviano (PN), Italy
[5]Department of Histopathology, Belluno City Hospital, 32100 Belluno, Italy
[6]Correspondence: hannon@cshl.org

Summary

Our knowledge of the transformation process has emerged largely from studies of primary rodent cells and animal models. However, numerous attempts to transform human cells using oncogene combinations that are effective in rodents have proven unsuccessful. These findings strongly argue for the study of homologous experimental systems. Here we report that the combined expression of adenovirus E1A, Ha-RasV12, and MDM2 is sufficient to convert a normal human cell into a cancer cell. Notably, transformation did not require telomerase activation. Therefore, we provide evidence that activation of telomere maintenance strategies is not an obligate characteristic of tumorigenic human cells.

Introduction

Neoplastic transformation occurs via a series of genetic and epigenetic alterations that yield a cell population that is capable of proliferating independently of both external and internal signals that normally restrain growth. For example, transformed cells show reduced requirements for extracellular growth promoting factors, are not restricted by cell-cell contact, and are often immortal (Paulovich et al., 1997; Hanahan and Weinberg, 2000). Through extensive studies of transformation processes in rodent models, it is known that tumor formation can be achieved by the activation of oncogenes and the inactivation of tumor suppressor pathways (Paulovich et al., 1997; Hanahan and Weinberg, 2000; Sherr, 1996). It has long been established that primary rodent cells can be transformed at detectable frequency by two oncogenic "hits," such as the combination of ectopic c-*myc* expression and constitutive activation of Harvey Ras (Ha-RasV12) (Land et al., 1983; Ruley, 1983). However, primary human cells have proven to be refractory to transformation by numerous combinations of cellular and viral oncoproteins, indicative of fundamental differences in requirements for transformation in human versus rodent cells (Sager, 1991; O'Brien et al., 1986; Stevenson and Volsky, 1986; Serrano et al., 1997).

Two major hypotheses have emerged as the underlying explanation for such differences. Primary human and murine cells respond to oncogene activation via homeostatic mechanisms that are proposed to enforce tumor suppression. For example, activation of oncogenes such as c-*myc* or adenovirus E1A sensitizes primary cells to apoptosis (Debbas and White, 1993; Lowe et al., 1994; Lowe and Ruley, 1993; Harrington et al., 1994; Hermeking and Eick, 1994). Hyperactivation of the ras oncogene or flux through the ras signaling pathway induces a state of terminal growth arrest which is phenotypically similar to cellular senescence (Serrano et al., 1997). In murine cells, the latter response can be bypassed by genetic alterations, which impair the p53 response. Indeed, cells lacking p53 or $p19^{ARF}$ can be transformed directly by activated ras (Kamijo et al., 1997; Serrano et al., 1996, 1997). In contrast, inactivation of the p53 pathway alone is insufficient to rescue human cells from ras-induced growth arrest (Serrano et al., 1997), suggesting that homeostatic responses in humans flow through multiple independent and redundant effector pathways.

A second characteristic that distinguishes primary human and murine cells is that the latter are easily immortalized (Blasco

SIGNIFICANCE

Activation of telomerase and consequently telomere maintenance is a common characteristic of human tumors. Existing models of human cancer cells, created by the introduction of defined genetic alterations, all include telomerase activation and/or telomere maintenance as an obligate component of the transformed phenotype. Here, we demonstrate that normal human cells can be converted into cancer cells capable of forming tumors in immunocompromised mice in the absence of telomerase activation or any other telomere maintenance strategy. This suggests that alterations in telomere biology must be viewed similarly to genomic instability as catalysts of transformation rather than as central components of the transformed phenotype.

et al., 1997a). Primary human cells rarely undergo spontaneous immortalization, indicating that the control of cellular lifespan is drastically different between these two cell types (Imam et al., 1997; Chin et al., 1999). This phenomenon can be partially attributed to telomere biology. Unlike the embryonic rodent fibroblasts which have served as common models for studies of transformation in vitro, primary human fibroblasts have relatively short telomeres and lack detectable telomerase activity (reviewed in McEachern et al., 2000).

The importance of telomerase in human tumorigenesis is supported by numerous observations. First, the majority of human tumors are telomerase-positive (Kim et al., 1994). Second, telomerase activation is sufficient to immortalize some primary human cells in culture (Bodnar et al., 1998; Counter et al., 1998; Wang et al., 1998). Third, telomerase is regulated by an oncogene, c-*myc*, which is activated in a high percentage of human cancers (Wang et al., 1998).

Previous reports have indicated that primary human fibroblasts and epithelial cells can be transformed by a defined combination of genetic elements, comprising the telomerase catalytic subunit, hTERT, the SV40 early region, and Ha-RasV12 (Hahn et al., 1999; Elenbaas et al., 2001, Hahn et al., 2002). Here we report an alternative model of human cell transformation. We show that coexpression of two oncogenes, adenovirus E1A and Ha-RasV12, is sufficient to enable primary human fibroblasts to grow in an anchorage-independent manner, a hallmark of in vitro transformation. However, this combination is insufficient to promote tumor formation in nude mice. Addition of a third oncogene, MDM2, can convert these fibroblasts into cells capable of forming tumors in vivo. Interestingly, both anchorage-independent growth in vitro and tumorigenesis in vivo occur in the absence of telomerase activation. Our results indicate that while telomerase activation is a common characteristic of human tumors, it is not an obligate element of the tumorigenic phenotype.

Results

Coexpression of E1A and Ha-RasV12 permits anchorage-independent growth

A defining characteristic of the transformed phenotype is a degree of independence from exogenous mitogenic signals. Many of these signals activate the ras pathway, and activating mutations of *ras* oncogenes or their upstream regulators often occur in human cancers (Barbacid, 1987; Webb et al., 1998). However, in both primary rodent and human cells, expression of the *ras* oncogene alone results in an irreversible growth arrest that is phenotypically similar to cellular senescence (Serrano et al., 1997; Lin et al., 1998). In rodent models, *c-myc* is capable of both bypassing ras-induced growth arrest and cooperating with activated *Ha-RasV12* to transform primary cells into tumorigenic cells (Land et al., 1983). However, combined expression of myc and activated ras in normal human cells not only fails to result in transformation, but also leads to an accelerated appearance of the senescent-like phenotype (data not shown).

Whereas numerous genetic alterations have been shown to bypass ras-induced growth arrest in murine cells, only very few have been shown to be capable of overriding this response in normal human cells. One of these is the ectopic expression of the adenovirus oncogene, E1A (Figure 1A) (Serrano et al., 1997; de Stanchina et al., 1998). In fact, coexpression of *E1A* and *Ha-RasV12* provided one of the first demonstrations of transformation by cooperating oncogenes in primary rodent cells (Ruley, 1983). Therefore, we tested whether combined expression of E1A and Ha-RasV12 could transform normal human primary fibroblasts.

One characteristic feature of transformed cells is their ability to grow in the absence of anchorage and, therefore, form colonies in semisolid media. Early passage normal human foreskin fibroblasts (designated BJ) expressing E1A or Ha-RasV12 individually failed to form colonies in soft agar. In contrast, cells expressing both E1A and Ha-RasV12 were able to form colonies in soft agar with an efficiency comparable to that seen with transformed human and rodent cells (Figure 1B). For human 293T cells, virtually all plated cells gave rise to colonies, compared to a range of 10%–30% for BJ/E1A/Ha-RasV12 (for example, see Figure 1C). In general, colonies generated by BJ/ER (E=E1A, R=Ha-RasV12) contain significantly fewer cells than those generated by 293T cells within the same time period.

The role of E1A in the transformation of primary human fibroblasts

E1A is a multifunctional protein that interacts with numerous cellular proteins involved in controlling proliferation. For example, E1A can bind members of the Rb family through conserved motifs designated CR1 and CR2 (Whyte et al., 1988; Harlow et al., 1986; Whyte et al., 1989). Through these interactions, E1A is able to modulate the activity of the E2F family of transcription factors, thus controlling genes required for entry into S phase (Wang et al., 1995; Paulovich et al., 1997; Sherr, 1996). The amino-terminus of E1A binds promiscuous transcriptional coactivators, including p300 (Dorsman et al., 1995; Wang et al., 1995; Goodman and Smolik, 2000). The amino-terminus also binds to the protein complex containing p400, a SWI2/SNF2 family member, and the c-Myc/pCAF-interacting protein, TRRAP (McMahon et al., 1998; Barbeau et al., 1994, Fuchs et al., 2001). This p400 binding region has been shown to be vital for E1A-mediated transformation in mouse cells (Fuchs et al., 2001). The carboxy-terminal region of E1A binds CtBP, a cellular protein, which has been proposed to recruit histone deacetylases (Goodman and Smolik, 2000).

In order to map the regions and interactions of E1A that are essential for its ability to cooperate with Ha-RasV12 in conferring anchorage-independent growth to primary human fibroblasts, we used a series of well-characterized deletion mutants for in vitro transformation assays (Samuelson and Lowe, 1997). Cells were coinfected with Ha-RasV12 and mutant E1A oncoproteins. While a truncated E1A protein consisting of only the amino-terminal 143 amino acids is unable to bind CtBP (Boyd et al., 1993; Meloni et al., 1999), this mutant is capable of cooperating with Ha-RasV12 for colony formation in soft agar with high efficiency (Figure 2). Expression of E1A-ΔCR2, a mutant incapable of binding pRb (Samuelson and Lowe, 1997), in combination with Ha-RasV12 invariably led to a senescence-like growth arrest. This result indicated that the interaction between E1A and Rb-family proteins is essential for transformation. Loss of the ability to bind p300 also compromised oncogene cooperation, as did deletion of residues 26–35, indicating that the ability to bind p400 is also critical. Western analysis of E1A mutants (Supplemental Figure S1 at http://www.cancercell.org/cgi/

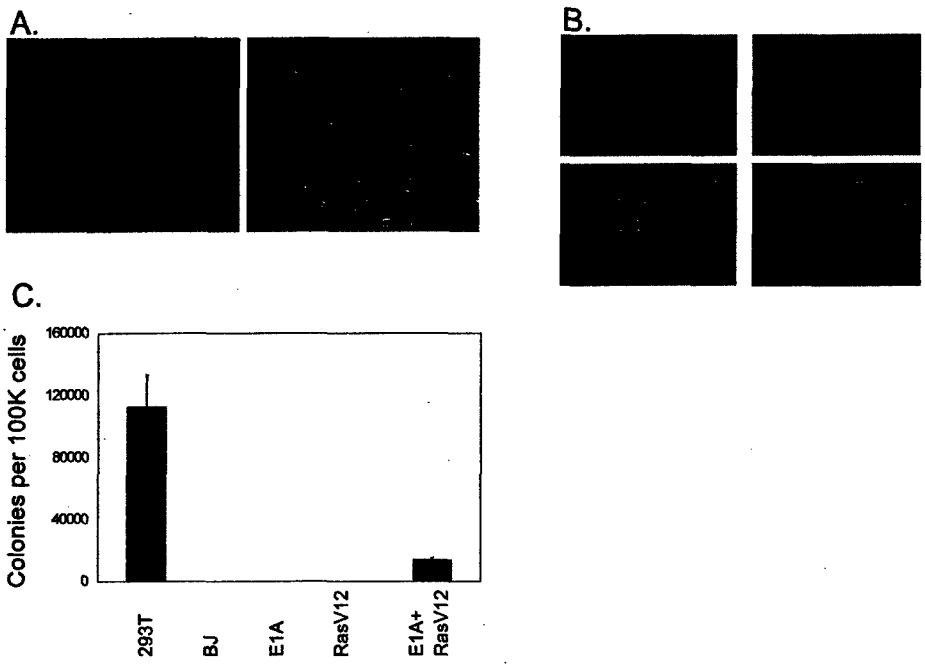


Figure 1. Transformation of normal human fibroblasts by E1A and Ha-RasV12

A: Normal human diploid fibroblasts (BJ) infected with a recombinant retrovirus that directs expression of Ha-RasV12 stain positively for expression of the senescence-associated β-galactosidase marker, whereas BJ fibroblasts coinfected with retroviruses for E1A (12s) and Ha-RasV12 expression do not stain for this marker and continue to proliferate.

B: BJ fibroblasts were infected with retroviruses for E1A or Ha-RasV12 expression alone or in combination and assayed for colony formation in semisolid media.

C: Colonies containing greater than 100 cells were counted from triplicate platings of cells with the indicated genotype to determine the rate of colony formation. Standard error from the mean is indicated.

content/full/2/5/401/DC1) suggested that each was expressed similarly to wild-type, and thus the defects in colony formation were not due to E1A not being adequately expressed. Expression of E1A-ΔCR2 mutant was confirmed only in transient infections, since the prevalence of cell death in ΔCR2/Ras coinfected cells prevented the establishment of a stable cell line. Considered together, these results suggest that E1A functions in human cell transformation through concerted effects on multiple cellular pathways that include Rb, p300, and p400.

E1A + Ha-RasV12-expressing cells fail to form tumors

Early and late passage BJ fibroblasts coexpressing E1A and Ha-RasV12 were tested for the ability to form tumors upon subcutaneous injection into immunocompromised mice. A total of 49 animals were injected in both flanks in a series of five independent experiments. Subject mice were either nude, SCID (beige), or nude mice that had been irradiated as a mean to suppress residual NK (natural killer) responses (Feuer et al., 1995). From a total of 98 injections, only a single tumor formed in a nude, nonirradiated mouse (Supplemental Table S1 at http://www.cancercell.org/cgi/content/full/2/5/401/DC1). This tumor arose after a substantially longer latency (10 weeks) than is normally observed using control cancer cell lines or transformed human 293T cells (~2 weeks), suggesting the possibility that a rare additional genetic alteration may have contributed to tumor formation in this individual case. Thus, we conclude that while

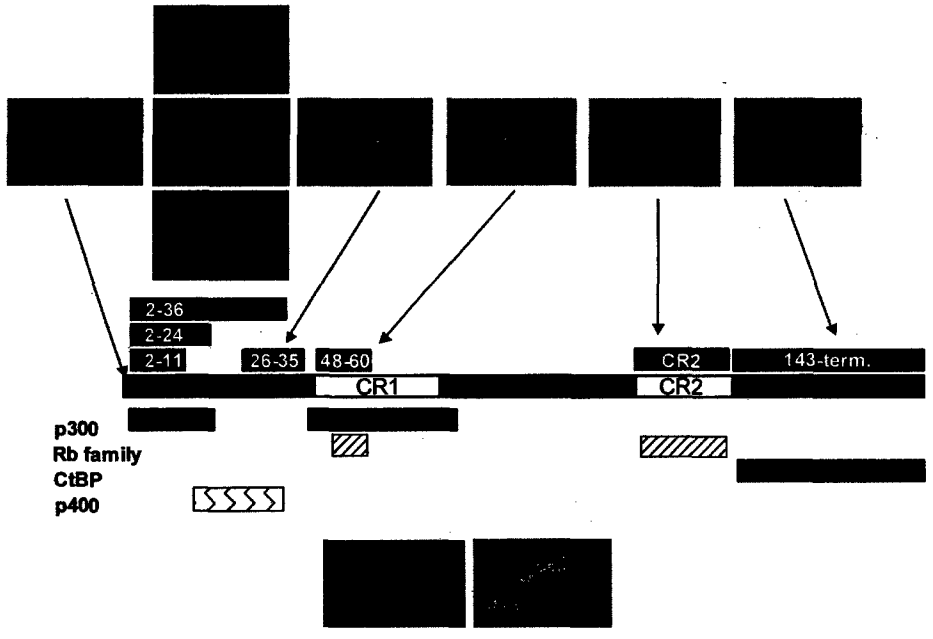


Figure 2. Functional analysis of the regions of E1A required for transformation in vitro

A series of well-characterized functional mutants of E1A were coexpressed in BJ fibroblasts with Ha-RasV12 and assayed for the ability to form colonies in semisolid media. Only the E1A-143 mutant, which is defective for the ability to bind CtBP, was capable of forming colonies in this assay, indicating that the ability of E1A to bind p300, p400, and Rb is critical for E1A-mediated transformation.

the combination of E1A and Ha-RasV12 is sufficient to permit anchorage-independent growth of normal human fibroblasts, this combination is insufficient for tumorigenesis in nude mice.

E1A, MDM2, and Ha-RasV12 transform normal human cells into tumor cells

Previous studies of E1A/Ha-RasV12-mediated transformation in primary mouse embryo fibroblasts (MEF) indicated that transformation mediated by this oncogene combination was much more efficient in the absence of p53 (Lowe and Ruley, 1993). In fact, tumors arising from E1A/Ras transformed MEF become apparent only after a long latency period and frequently lack a functional p53 pathway. Interestingly, immunohistochemical analysis of the single tumor produced by the BJ fibroblasts expressing E1A/Ha-RasV12 showed a strong accumulation of nuclear p53; however, results of SSCP analysis excluded the possibility of p53 gene mutations (data not shown).

Accumulation of wild-type p53 is a common feature of human sarcoma, the type of tumor derived from fibroblast precursors. In addition, these tumors often show overexpression of MDM2 (Dei Tos et al., 1997), indicating that negation of p53 function occurs often through mechanisms other than p53 gene mutation. Notably, the tumor that resulted from the E1A/Ha-RasV12-expressing fibroblasts was negative for the expression of $p19^{ARF}$, an upstream regulator of MDM2 (by immunohistochemistry, data not shown), whereas the preinjection population of engineered fibroblasts expressed $p19^{ARF}$ abundantly. Guided by these observations, we tested whether negation of the p53 pathway via enforced expression of MDM2 could contribute to the transformation of normal human fibroblasts by E1A and Ha-RasV12.

BJ cells at different passage numbers (see Experimental Procedures for details) were simultaneously coinfected with three retroviruses that direct the expression of E1A, Ha-RasV12, and MDM2 with each retrovirus bearing a different drug selection marker. Control cells were prepared by replacing individual oncogene-expressing viruses with an empty vector bearing the same selection marker. These triple-infected populations were simultaneously coselected with puromycin, hygromycin, and neomycin for ten days and then either plated into soft agar (Figure 3) or injected into immunocompromised mice (Figure 4). Expression of the ectopically expressed oncogenes was confirmed by Western blot (not shown). Cell populations expressing E1A/Ha-RasV12/MDM2 formed colonies in soft agar with higher efficiency than BJ/E1A/Ha-RasV12 (Figure 3B). Moreover, the triple-infected cells were able to generate tumors when injected subcutaneously into immunocompromised in mice (Figure 4A and Table 1). Tumors grew to a size at which the animals had to be sacrificed within a period of three to six weeks after injection, a latency comparable to that seen with control human cancer cell lines or with transformed 293T cells (Figure 4B). Tumor formation was also observed when E1A was substituted by the C-terminal deletion mutant E1A-143 (data not shown). Histological and immunohistochemical analyses of ERM-derived tumors confirmed the human origin of the tumor cell population and indicated that the neoplasias have features of sarcoma. Moreover, immunohistochemistry confirmed the widespread and strong expression of E1A, Ras, and MDM2 oncogenes (Figure 4C).

Cell populations remained polyclonal throughout drug selection in vitro and tumorigenesis in vivo, as revealed by Southern blotting analysis (Supplemental Figure S2 at http://www.cancercell.org/cgi/content/full/2/5/401/DC1). These results argue against the possibility of selection for rare genetic events during tumor formation and support the notion that the combined expression of E1A, MDM2, and Ha-RasV12 is sufficient for the transformation of normal human fibroblasts into tumor cells.

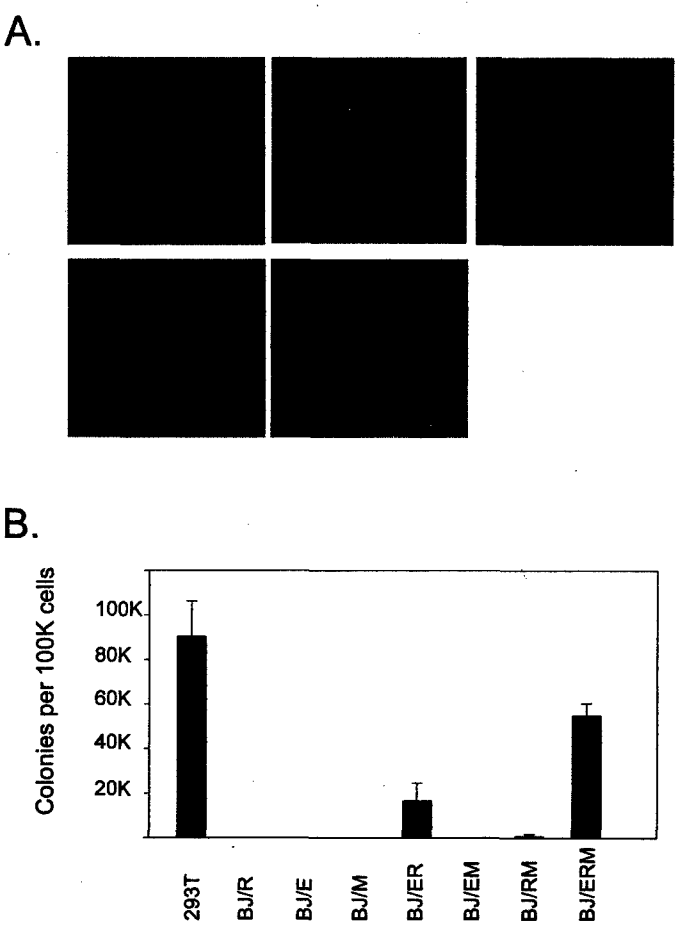


Figure 3. MDM2 cooperates with E1A and Ha-RasV12 to enhance colony formation in vitro

A: BJ fibroblasts were infected with retroviruses to direct the expression of three genes, E1A (E), Ha-RasV12 (R), and MDM2 (M), alone and in combination, and plated into semisolid media. Notably, cells engineered to express all three genes simultaneously (ERM) formed robust colonies similar to those produced in the 293T control.

B: Colonies containing greater than 100 cells were counted from triplicate platings of cells with the indicated genotype in order to determine the rate of colony formation. The standard error from the mean is indicated. ERM cells form colonies at a rate greater than ER cells and more comparable to the control 293T cell line.

Human fibroblasts transformed by E1A/MDM2/Ha-RasV12 lack telomerase activity

Cell immortalization has been posited as a landmark occurrence in the transformation of a normal cell into a cancer cell. Indeed, most human cancers are telomerase-positive, an indirect indication that these cells have acquired a mechanism for both telomere maintenance and extension of proliferative capacity (Kim et al., 1994). In previous reports, transformation of normal human cells absolutely required activation of telomerase via ex-

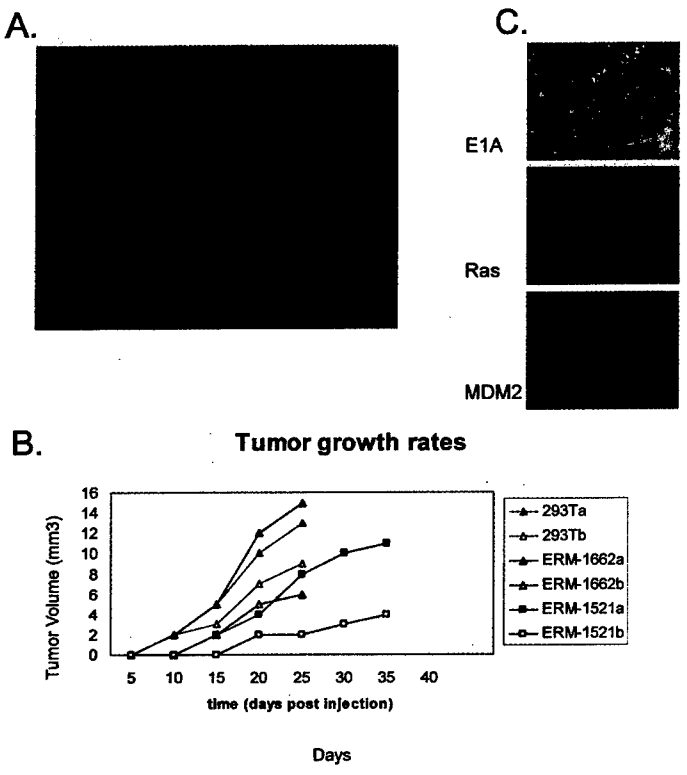


Figure 4. Conversion of BJ fibroblasts into tumor cells by combined expression of E1A, Ha-RasV12, and MDM2

A: Examples of immunocompromised mice (nude, no γ irradiation) that have been injected with either control BJ fibroblasts or BJ cells that have been engineered to express E1A (E), Ha-RasV12 (R), and MDM2 (M).

B: Tumor growth rates from two representative mice (ERM-1662 and ERM-1521) injected in both flanks (a, left; b, right) with BJ/ERM fibroblasts are compared to tumor growth rates in a mouse that had been injected in both flanks with E1A-expressing 293T cells, as indicated.

C: Immunohistochemistry staining of ERM tumor samples with antibodies to E1A, Ha-Ras, and MDM2, verifying that the tumors were in fact derived from the injected cells

Table 1. Formation of subcutaneous tumors in nude mice by human fibroblasts expressing E1A, Ha-RasV12, and MDM2

Tumor formation in immunodeficient mice: E1A + MDM2 + Ha-RasV12	
Cells	Number tumors/ number injected
293T	6/6
BJ	0/10
BJ/E1A + Ha-RasV12	0/6
BJ/E1A + MDM2	0/5
BJ/E1A + MDM2 + Ha-RasV12	34/48
BJ/E1A-143 + MDM2 + Ha-RasV12	6/6
Tumor formation: Additional human fibroblast strains	
Cells	Number tumors/ number injected
BJ/E1A + MDM2 + Ha-RasV12	12/12
HSF43/E1A + MDM2 + Ha-RasV12	2/8
WI-38/E1A + MDM2 + Ha-RasV12	7/8
Detroit551/E1A + MDM2 + Ha-RasV12	7/7

For each injection, 5×10^6 cells of the indicated populations were injected subcutaneously in a volume of 100 μl. Mice were sacrificed when the tumors reached a diameter of 1 to 1.2 cm or after 16 weeks of monitoring. The top and bottom portions of the table represent two independent series of experiments.

pression of the limiting catalytic subunit, hTERT (Hahn et al., 1999; Elenbaas et al., 2001, Hahn et al., 2002). We previously showed that E1A, Ha-RasV12, and MDM2 were individually incapable of activating telomerase in normal human fibroblasts or epithelial cells (Wang et al., 1998). We therefore tested the possibility that we had transformed normal human cells into cancer cells in the absence of telomerase activation.

Telomerase activity was easily detected in 293T cells using the TRAP assay (Kim et al., 1994; Wright et al., 1995). As few as ten 293T cells were capable of yielding a strong positive signal in our assays. As expected, BJ fibroblasts are telomerase-negative. We similarly fail to detect telomerase activity in BJ cells that have been engineered to express E1A, Ha-RasV12, and MDM2 (BJ/ERM) (Figure 5A). We conclude that BJ/ERM cells are telomerase-negative, or contain at least 1000-fold less telomerase activity than do 293T cells at the time they are injected into immunocompromised mice.

It is interesting to note that BJ/ERM cells, although able to form colonies in soft agar and tumors in nude mice, are not immortal and, if maintained in culture for an extended period of time (40–50 generations), undergo a "crisis phase" characterized by dramatically reduced proliferation and adoption of a senescent phenotype. Few BJ/ERM cells eventually survive this phase, and these cells become telomerase-positive (Figure 5A, ERM P.C.). This behavior is suggestive of a "telomere crisis" as a consequence of the absence of a telomere maintenance program. This hypothesis is supported by an examination of telomere dynamics in BJ/ERM cells. Telomeres shrink continuously as cells are passaged in culture, reaching an average length of ~3 kb prior to entering a crisis phase from which the population emerges with detectable telomerase activity (Figures 6A and 6B).

Tumors derived from E1A/MDM2/Ha-RasV12 lack telomerase activity

Since ERM-engineered fibroblasts were telomerase-negative at the time of injection into mice, we were curious to ascertain the telomerase status of resultant tumors and to determine whether telomerase activation was a requirement for tumorigenesis. Telomerase activity was measured utilizing the standard TRAP assay described above on tissue sections obtained from the ERM tumors. Whereas a tissue sample from a human tumor produced a robust signal indicative of telomerase activity in a TRAP assay, lysates from ERM tumor tissues were telomerase-negative (Figure 5B). In order to verify that this negative result was not due to the presence of an inhibitory component within the tissue lysate, we performed a mixing experiment with lysate from 293T cells. When the 293T cell and ERM tumor lysates were mixed in a TRAP reaction, the result was positive, indicating that there was no inhibitory component within the tumor lysate, and thus the tumors were below detectable limits for telomerase activity (Figure 5B).

To verify the forgoing result, we used an independent experimental strategy. In human cells, and in particular in human fibroblasts such as BJ, telomerase activity correlates with the

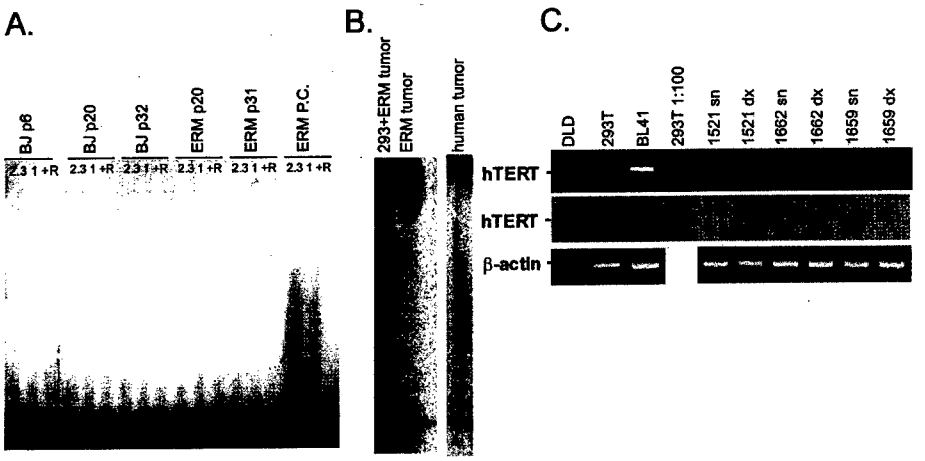


Figure 5. BJ/ERM cells are telomerase-negative upon injection into nude mice

A: The TRAP assay was used to detect telomerase activity in uninfected BJ cells and BJ/ERM cells at various passages. At the time of injection (p20), ERM cells are telomerase-negative. However, after continuous passaging in culture, these cells undergo an event similar to crisis, a point at which many cells undergo apoptosis or senescence. Cells that emerged from this crisis event become telomerase-positive (ERM P.C.). The indicated protein concentrations (μg) of S-100 extract were used. The highest protein concentration, 2.3 μg, was also pretreated with RNase (R) as a negative control.

B: Tumors were recovered from mice injected with BJ/ERM cells and assayed for the presence of telomerase activity using the TRAP assay. To test whether tissue extracts contained inhibitors of any step of the procedure, we mixed lysate derived from 1,000 telomerase-positive 293T cells with the tumor extract. This produced a positive signal. The figure shows one representative tumor sample. For comparison, a similar telomerase assay performed using a mass-equivalent portion of lysate from a human breast tumor is shown.

C: RT-PCR was used to detect hTERT expression in BJ-ERM-derived tumors (tumors 1521, 1662, and 1659; left, sn^-; right, dx^-). Expression was tested by using two primer pairs that would direct the specific amplification of hTERT and not mouse TERT that might be present from contaminating murine cells within the tissue sample. β-actin served as an internal control. Sensitivity of the assay was increased by performing a Southern blot of the PCR reactions for hTERT. 293T, DLD colon cancer, and BL41 lymphoma cell lines were used as positive controls. 293T 1:100 represents the PCR product of 293T cell line diluted 1:100, in order to have an approximate quantification of the signal. The figure shows the result of 40 cycles of amplification for hTERT and 20 cycles for β-actin.

expression of the telomerase catalytic subunit, hTERT (Meyerson et al., 1997; Bodnar et al., 1998). We used an RT-PCR strategy to search for hTERT expression in BJ/ERM tumor specimens. Expression was tested using two independent primer pairs that were chosen for their ability to specifically amplify human TERT without amplifying mouse TERT that might be present from contaminating murine cells in the tumor sample. β-actin mRNA served as an internal control. Ethidium bromide gel staining showed that hTERT mRNA was easily detectable in RNA derived from human cancer cell lines, but BJ/ERM tumors were negative (Figure 5C). To increase the sensitivity of our assay, we performed Southern blots of the PCR reactions. After Southern analysis, one out of six tumors analyzed, sample 1659sn, showed weak hTERT expression. This signal was detectable only with an exposure at which the signal of the positive control cells had reached saturation. The expression of hTERT in this tumor was independently confirmed by increasing the number of PCR cycles to 50 (see Figure 9B). Considered together, these data suggest that BJ/ERM cells were competent for tumor formation in the absence of telomerase activity and that activation of telomerase can occur as a late event during tumor progression.

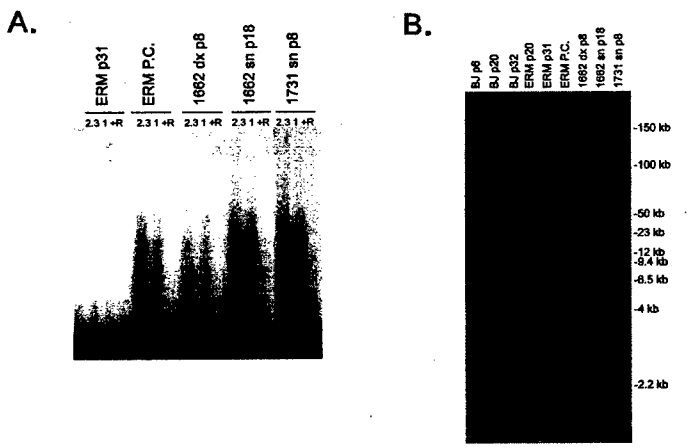


Figure 6. Telomere dynamics in ERM-transformed cells before and after tumor explant

A: The TRAP assay was used to detect telomerase activity in cells explanted from ERM tumors and compared to ERM cells that had been in continuous culture. The explanted cell lines (1662dxp8, 1662snp18, and 1731snp9) were telomerase-positive, similar to what was seen in ERM cells that had been continuously passaged in culture until they underwent crisis (ERM P.C.).

B: Telomere length in uninfected BJ, BJ/ERM, and ERM tumor explants were analyzed by TRF using pulse-field electrophoresis. As predicted, the telomeres of uninfected BJ and BJ/ERM become shorter as the cells are passaged in vitro. Interestingly, the telomeres of both ERM P.C. and explanted cell lines (1662dxp8, 1662snp18, and 1731snp9) had become extremely short irrespective of the fact they were telomerase-positive.

Upon explantation into culture, BJ/ERM tumor cells, similar to late passage ERM, undergo a crisis event, which is marked by cellular senescence and apparent cell death. In contrast, explantation of tumors generated with 293T control cells did not produce a similar outcome. Instead, these cells proliferate robustly. Following this crisis event, few BJ/ERM tumor cells emerge to form a sustainable population. In contrast to early passage BJ/ERM cells and to BJ/ERM tumor samples, and similar to postcrisis late passage BJ/ERM, surviving tumor cells have become telomerase-positive (Figure 6A). The forgoing is suggestive of a "telomere crisis" possibly related to the lack of a telomere maintenance program in the tumor mass, a crisis that could be compensated by an in vitro selection of a cell population with activated telomerase.

In accord with this hypothesis, TRF assays and telomeric FISH confirmed that continuous telomere erosion occurred during transformation in vitro and tumor formation in vivo (Figures 6B and 7). Telomeres in early passage BJ cells averaged ~7 kb in length. These became depleted as BJ cells were engi-

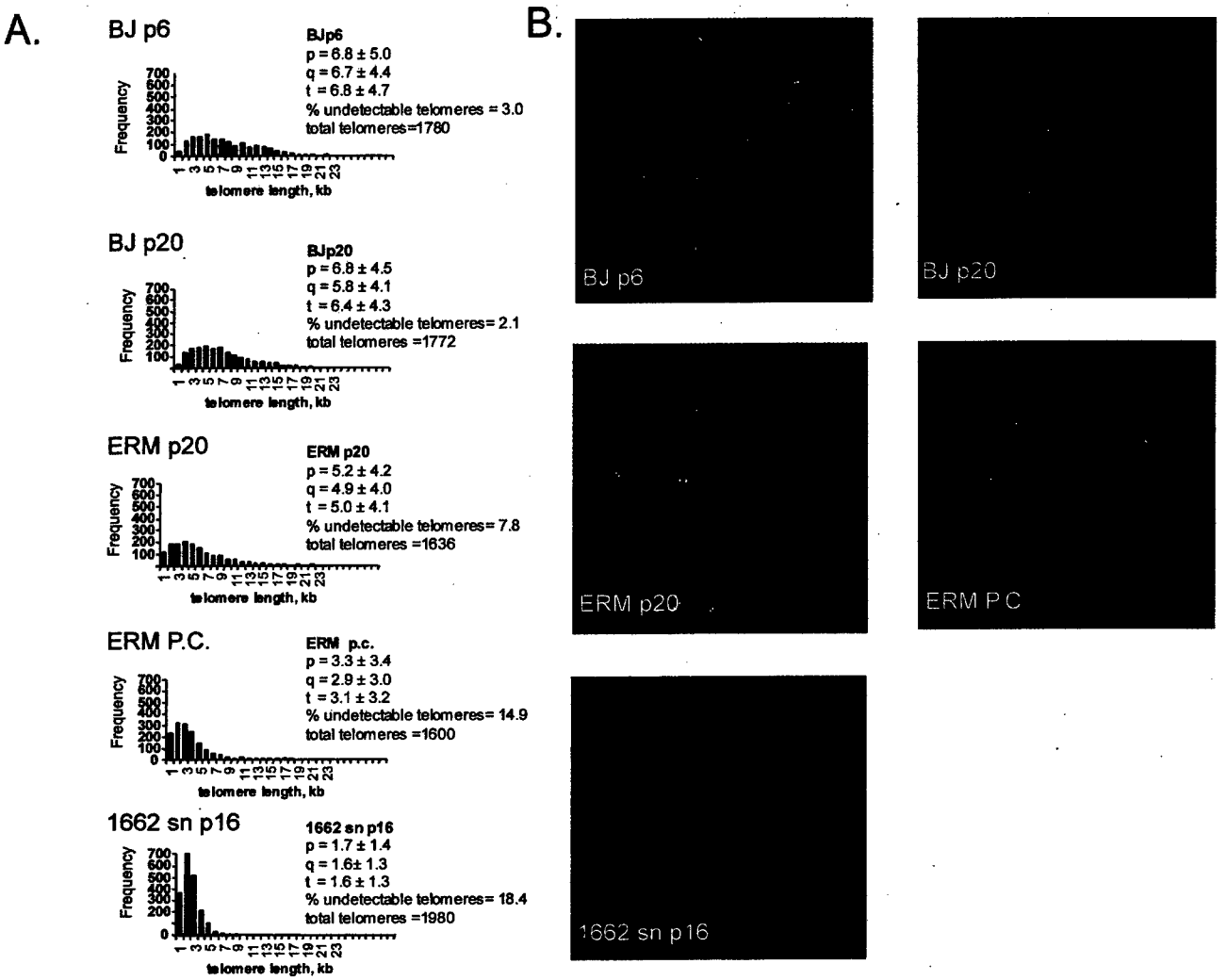


Figure 7. Quantitative FISH analysis of ERM cells preinjection and postexplant

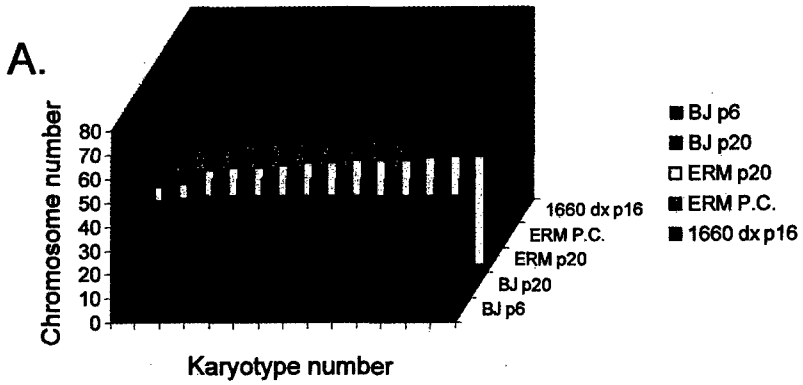
A: Telomere length distribution of BJ, BJ/ERM, and BJ/ERM tumor cells at different passages as determined by quantitative FISH (Q-FISH) using a fluorescent Cy-3 labeled telomeric peptide nucleic acid (PNA) probe. Average telomere length in kbps and standard deviation, as well as the total number of telomeres analyzed for each cell culture, are indicated. The percentage of undetectable telomeres using Q-FISH is also indicated.

B: Representative Q-FISH images of metaphases from BJ, BJ/ERM, and BJ/ERM tumor cells hybridized with a telomeric PNA probe. We note a significant decrease in telomeric signal, as well as the increased chromosomal instability and increased aneuploidy in later passage ERM cells and in BJ/ERM tumor-derived cells, 1662sn. Blue: DAPI; yellow dots: telomeres.

neered to express oncogenes and were passaged in vitro to an average of 5 kb at passage 20 and 3.1 kb in BJ/ERM after crisis occurred in vitro. Consistent with an apparent lack of a telomere maintenance strategy, telomere depletion continued during tumor formation in vivo such that explanted cell cultures had extremely short telomeres, averaging 1.6 kb with 18% of chromosome ends lacking detectable telomeric DNA (Figure 7). These results rule out the possibility that BJ/ERM tumors have activated the recombination-based pathways of telomere maintenance (ALT) (Dunham et al., 2000; Bryan and Reddel, 1997; Hoare et al., 2001; Bryan et al., 1997). Interestingly, cells explanted from ERM tumors that had become telomerase-positive in vitro (Figure 6A) maintained their ability to form tumors in nude mice and did so at rates similar to those observed for primary ERM cells (data not shown).

The karyotypes of explanted BJ/ERM cells reveal chromosomal abnormalities characteristic of telomere depletion

As noted above, BJ cells are engineered to express E1A, HaRasV12, and MDM2 through simultaneous coinfection. Since these cells have not undergone prolonged expansion in the presence of any individual oncogene in culture, it is not surprising to find that the karyotypes of the engineered cells are normal prior to injection into mice (Supplemental Figure S3A at http://www.cancercell.org/cgi/content/full/2/5/401/DC1). Examination of cells that are explanted into culture following tumor formation, however, reveals numerous chromosomal abnormalities (Figure 8, Supplemental Table S2, and Supplemental Figure S3B). In virtually every metaphase, we noted the presence of dicentric chromosomes lacking telomeres at the fusion point that apparently formed via end-to-end fusion of TTAGGG-



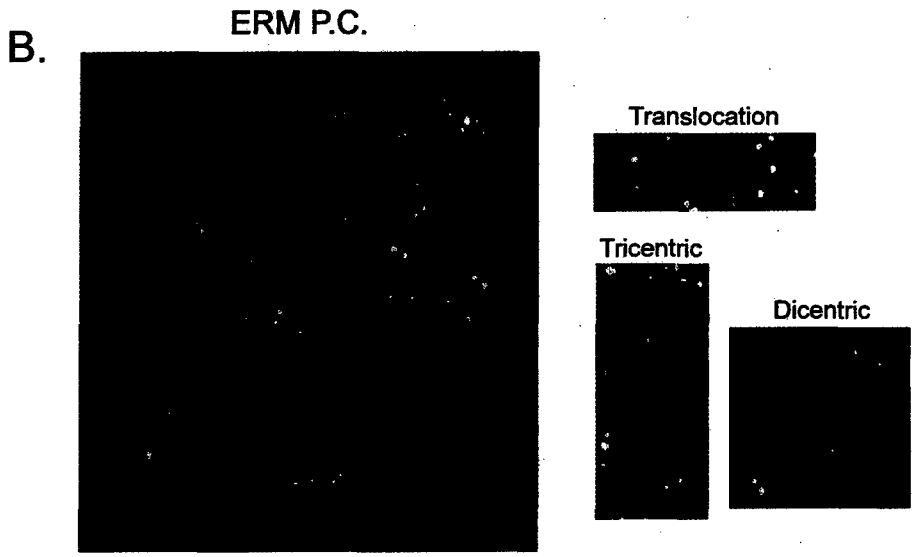


Figure 8. Comparative karyotype analysis of BJ, BJ/ERM, and BJ/ERM tumor-derived cells

A: Karyotype analysis of BJ, BJ/ERM, and BJ/ERM tumor cells at different passages is shown. For each culture, 15 metaphases were evaluated. Notice that the tumor-derived cells were severely aneuploid compared with BJ cells at two different passages. ERM p20 and ERM P.C. cells show a modest aneuploidy.

B: Representative images of cytogenetic alterations detected in metaphases from BJ/ERM explanted tumor cells after staining with DAPI and a fluorescent Cy-3-labeled telomeric PNA probe. TRANS, translocation; DICENT, dicentric; TRICENT, tricentric; blue, chromosome DNA stained with DAPI; yellow and white dots, TTAGGG repeats.

depleted telomeres. In some metaphases, we also find ring chromosomes (Supplemental Figure S3B). In addition, these cells showed a very marked aneuploidy as indicated by aberrant number of chromosomes in more than 50% of the metaphases analyzed, also in agreement with aberrant mitosis as a consequence of severe telomeric dysfunction (Figure 8). These types of genetic abnormalities are a characteristic outcome of telomere depletion and are similar to those seen in the karyotypes of *Terc*$^{-/-}$ mice (Blasco et al., 1997b; Nanda et al., 1995). Considered together, the results of telomerase detection assays, telomere restriction fragment analyses, and cytogenetic examination of explanted tumor cells strongly suggest that combined expression of E1A, Ha-RasV12, and MDM2 is capable of transforming normal human cells into human tumor cells in the absence of direct telomerase activation or alternative mechanisms of telomere maintenance.

Multiple human primary fibroblasts can be transformed by coexpression of E1A/MDM2/Ha-RasV12

In order to ascertain whether the transforming potential of the ERM combination relied on the relatively long telomeres found in early passage BJ fibroblasts, populations of cells at different passage numbers were infected and tested for anchorage-independent growth and tumor formation (see Experimental Procedures for details). In all the conditions tested, both early and late passage BJ/ERM invariably produced colonies in soft agar and tumors in immunocompromised mice.

Finally, to verify that E1A/MDM2/Ha-RasV12-mediated transformation is not unique to BJ fibroblasts, we assessed the validity of our transformation model in several additional human primary fibroblasts, including HSF43, WI-38, Detroit 551, and SF68 as well as in human primary mesodermal cells, HMSC. Upon coexpression of E1A/MDM2/Ha-RasV12, all were capable of anchorage-independent growth in soft agar (not shown). Efficiencies of colony formation and rates of colony growth were similar to those seen with BJ/ERM cells. Furthermore, these triple-infected fibroblasts were capable of tumor formation when injected into immunocompromised mice (Table 1 and Figure 9A). Similar to BJ/ERM, HSF43/ERM, WI-38/ERM, and DET551/ERM fibroblasts were telomerase-negative and the majority of the tumors derived from these cells failed to show telomerase expression by hTERT-RT-PCR analysis. Only 2 out of 8 tumors tested showed a faint hTERT band following 50 cycles of amplification (Figure 9B). This confirms that also in HSF43, WI-38, and DET551 fibroblasts, the ERM combination does not require telomerase activation to confer tumorigenic potential.

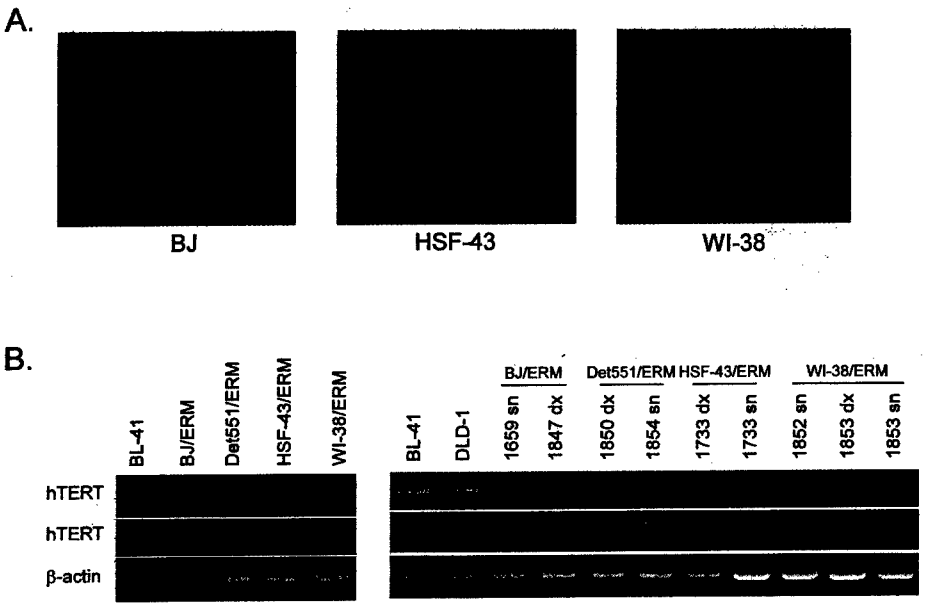


Figure 9. Multiple human fibroblast strains can be transformed in the absence of direct telomerase activation

A: Examples of immunocompromised mice (nude, no γ irradiation) injected with control fibroblasts expressing E1A + Ha-RasV12 or fibroblasts engineered to express the oncogene combination of E1A + Ha-RasV12 + MDM2. As had been seen with BJ fibroblasts, additional human primary fibroblasts (HSF43, WI-38, and Detroit551) became tumorigenic upon expression of the combination of ERM, while the combination of ER alone was unable to result in tumorigenesis.

B: RT-PCR analysis for hTERT expression in BJ, Detroit551, HSF-43, and WI-38 primary cells expressing E1A + Ha-RasV12 + MDM2 (left panel). RT-PCR analysis for hTERT expression in BJ/ERM tumors (1659 and 1857), Det-551/ERM tumors (1850, 1854), HSF-43/ERM tumors (1733 left and right tumor), and WI-38/ERM tumors (1852 and 1853 left and right tumor) (right panel). BL41 and DLD1 cell lines were used as positive controls. The figure shows the result of 50 cycles of amplification for hTERT and 20 cycles for β-actin.

Discussion

Primary rodent cells and animal models have made invaluable contributions to our understanding of neoplastic transformation and of the biology of oncogenes and tumor suppressors. However, it is clear that these models do not perfectly recapitulate the process of tumor development in humans. An early indication of this fact was the inability of human cells to become transformed by the same combinations of oncogenes that could transform a variety of normal rodent cells. Recently, the ability to elicit transformation via specific genetic manipulations was extended to normal human cells (Hahn et al., 1999; Elenbaas et al., 2001, Hahn et al., 2002). This has created the opportunity for the development of a variety of defined human cancer models to be used for a detailed study of the cellular pathways that are required for the transformation of normal human cells, and ultimately, to an understanding of any differences in requirements for the transformation of human cells versus those of model organisms. Such information could provide critical insights as rationally designed anticancer therapies move from successful applications in animal models to use in humans.

Here we report that primary human fibroblasts can be transformed into cancer cells by combined expression of the adenovirus E1A, Ha-RasV12, and MDM2. As in previous models of human cell transformation, we make use of a combination of viral and cellular oncoproteins that act in a transdominant fashion to alter cellular physiology and achieve tumorigenic growth. In accord with previous reports, we show that transformation requires negation of both the Rb and p53 tumor suppressor pathways. Through genetic analyses, we have also identified requirements for interaction with p300 and p400. Both of these cellular proteins are also targeted by SV40 large T-antigen, which is a critical element of the transformation model reported by Weinberg and colleagues (Hahn et al., 1999; Elenbaas et al., 2001). However, recent reports suggest that these are not critical functions of large T, at least in the presence of small t-antigen (Hahn et al., 2002).

One striking difference between our results and those reported previously is that in our transformation model, we find no requirement for telomerase activation to achieve either anchorage-independent growth in vitro or tumor formation in vivo. In fact, consistent with their lack of telomerase or other telomere maintenance strategies, our in vitro-engineered tumor cells show continuous erosion of telomeric repeats. This ultimately leads to genetic instability that is typified by our observation of numerous chromosome end-to-end fusions and pronounced aneuploidy in cells explanted from tumor tissue.

The majority of human cancer cells are reported to be telomerase-positive (Kim et al., 1994), and this is long been considered a strong indication that the ability to maintain telomeres is an important step in the development of human cancer. However, it is still debated whether the widespread presence telomerase activity in human tumors is a reflection of a selective expansion of a telomerase-positive stem cell or a selection for a mechanism of telomere maintenance during cancer progression.

Our results are consistent with a model in which telomere maintenance is not essential for transformation, per se, but instead serves as a catalyst of tumorigenic conversion and tumor progression. Mouse models have shown that alterations in telomere biology may contribute to tumorigenesis in two ways. We and others have previously reported that telomere shortening triggers growth arrest and/or apoptosis, as well as chromosomal end-to-end fusions, leading to premature aging phenotypes in the context of the telomerase-deficient mice. These phenotypes can be rescued by telomerase activation (Lee et al., 1998; Herrera et al., 1999, 2000; Samper et al., 2001). Thus, telomere shortening during the presumably prolonged course of natural tumor development may antagonize tumorigenesis. Indeed, $Terc^{-/-}$ mice are more resistant to chemical carcinogenesis and to spontaneous tumor development in both p16/p19ARF and APC^{min} mutant backgrounds (Greenberg et al., 1999; González-Suárez et al., 2000; Rudolph et al., 2001). However, exhausted telomeres can also compromise chromosome

integrity. In fact, in a $p53^{+/-}$ background, $Terc^{-/-}$ mice show higher levels of chromosomal instability and a higher incidence of cancer, suggesting that telomere depletion can actually act as a prooncogenic factor under certain conditions (Chin et al., 1999).

The data presented here suggest that, while changes in telomere biology are very likely to be important for the course of natural tumor development, telomere maintenance is not an absolute requirement for the creation of human cancer cells by acute alteration of oncogenes and tumor suppressors. Rather, in our human transformation model, the activation of telomere maintenance strategies becomes important only during prolonged expansion of tumor cells to restore genomic stability to an extent that permits cell survival. Our data are in agreement with observations in a number of human cancers where the frequency and intensity of telomerase activation correlates with tumor grade/stage (Chadeneau et al., 1995; Hiyama et al., 1995; Albanell et al., 1997; Tang et al., 1998; Ebina et al., 1999; Yan et al., 1999). Indeed, our ERM system can be considered as a human complement to a mouse model recently developed by DePinho and colleagues. They have shown that the absence of telomerase in $Terc^{-/-}Apc^{min}$ mice increases the frequency of early stage intestinal adenomas but decreases the multiplicity and size of later stage lesions. They further suggest that chromosome instability arising from telomere depletion may promote early stage carcinogenesis, but that acquisition of telomere maintenance strategies is important for tumor progression (Rudolph et al., 2001).

Using oncoprotein mutants and genetic complementation, we find that inactivation of the Rb and p53 tumor suppressor pathways is critical for this transformation process. Furthermore, we find that the ability of E1A to target p300 and p400 is essential for its ability to function as a human oncogene. It will also be of interest to determine whether MDM2 contributes to the transformation of human cells solely through its ability to antagonize p53 or also via effects on additional cellular pathways.

The war on cancer is predicated on the notion that increased understanding of the biology of cancer cells might reveal an "Achilles heel" that can be exploited as an effective and specific therapeutic target. The use of rodent cell culture and of animal models have been the most informative vehicles in the drive toward this goal. However, the availability of defined human cell transformation models will allow us to build toward a complete understanding of the biological pathways that must be altered to achieve tumorigenic conversion of normal cells.

Experimental procedures

Cells

The following human primary cells were used for the experiments described in the text. We defined passage number 1 as the passage at which the cells were provided from ATCC. All the cells were maintained at a subcultivation ratio of 1:2 every 3 days.

BJ are normal human foreskin fibroblasts (ATCC catalog CRL-2522). They were provided by ATCC at population doubling 22 and are described to have the capacity to reach 80 ± 10 PD. In our hands, these cells cease proliferation at around passage 45. Most of the experiments described in the text were done using BJ infected at passage 16 and injected at passages 20, 25, and 30. However, experiments were also performed at earlier and later passages. In particular, BJ cells were infected at passage 8, 16, and 22. Cells infected at passage 8 were injected into mice at passage 12 and 20. Cells infected at passage 16 were injected at passage 25 and 30, while cells infected at passage 22 were injected at passage 25 and 33.

WI-38 are normal embryonic lung fibroblasts (ATCC catalog CCL-75). These cells have a finite lifespan of 50 ± 10 PD and were provided by ATCC at PD24. These cells were infected at passage 6 and 10 and injected into mice at passage 14, 15, and 18.

Detroit 551 are normal human fetal fibroblasts (ATCC catalog CCL-110) provided by ATCC around PD 10 and reported to have a finite lifespan of 25 additional serial passages. These cells were infected at passage 6 and 10 and injected into mice at passage 14–15 and 18.

HSF43 are normal human foreskin fibroblasts originally isolated at the Los Alamos National Laboratory. The original PD of these cells was unknown but in our hands they cease proliferation after 40–50 passages. These cells were infected at passage 17 and 20 and injected at passage 24 and 27.

HS68 are normal human foreskin fibroblasts (ATCC catalog CRL-1635) that were received from ATCC at PD 16. These cells can be propagated for 40–50 passages. They were infected at passage 11 and 17.

HMSC 7214 are normal human mesenchymal stem cells obtained from Poietics-Biowhittaker at passage 2 (catalog PT-2501). They were derived from a 19-year-old Caucasian male and their lifespan has not been determined. These cells were infected at passage 6.

Cell culture conditions

BJ normal human foreskin fibroblasts were maintained in minimum essential medium with Earle's salts (MEM) supplemented with nonessential amino acids (NEAA) and 10% fetal bovine serum (FBS) (Gibco BRL). 293T, Detroit 551, WI-38, HSF43, and SF68 cells were maintained in Dulbecco's modified Eagle culture medium (DMEM), supplemented with 0.01% sodium pyruvate and 10% FBS. The same lot of serum was used throughout the experiments. HMSC human primary mesodermal cells (Poietics, BioWhittaker) were grown in MSCGM synthetic medium (Poietics, BioWhittaker). All cells were cultured at 37°C in the presence of 5% CO2. All human primary fibroblasts were maintained at a subcultivation ratio of 1:2 every 3 days.

Retroviral infection

pBABE-Puro Ha-rasV12, WzI-Neo E1A 12s, pHygroMaRX mdm2, and corresponding empty retroviral vectors were used to singularly transfect the amphotropic packaging cell line LinX-A (Hannon et al., 1999). Transfection was performed by the calcium phosphate method. At 72 hr posttransfection, viral supernatants were collected, filtered, supplemented with 4 μg/ml polybrene, and combined in order to obtain the oncogene combinations described in the text. In cells where only one or two oncogenes were used to infect the primary cells, corresponding empty vectors replaced the omitted oncogenes so that infected cells were equally resistant to all the selection drugs used (hygromycin, puromycin, and neomycin). The proper viral mix was then used to infect human primary fibroblasts (BJ, Detroit 551, WI-38, HSF43, SF68) and human primary mesodermal cells (HMSC). After infection, cells were selected with a combination of hygromycin (50 μg/ml), puromycin (1 μg/ml), and neomycin (300 μg/ml) for 7 days. Effective infection was confirmed by Western blot analysis.

Western blot analysis

Western blotting was performed essentially as described by Harlow and Lane (1999). 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 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were incubated with the following mouse monoclonal antibodies: E1A-specific M58 and M73 antibody—the latter recognizes an epitope retained in all E1A mutants studied (Samuelson and Lowe, 1997); c-Ha-ras (OP23) (Oncogene Research Products); mdm2-spcific antibody (4B2) (a kind gift from A. Levine); bcl2 (C2) (Santa Cruz); p53 (DO-1) (Santa Cruz). Immune complexes were visualized by secondary incubation with a sheep anti-mouse HRP-conjugated secondary antibody (Amersham). Blots were developed by enhanced chemiluminescence (Amersham).

Anchorage-independent growth

Human primary fibroblasts uninfected, infected with a control empty virus, or expressing different oncogenes (E1A, Ha-rasV12, mdm2), alone or in combination, were analyzed for anchorage-independent growth in semisolid media. Approximately 10^5 cells were plated in 0.3% low melting point aga-

rose/growth media onto 60 mm dishes with a 0.5% agarose underlay. Fresh top agar was added weekly. Colonies were photographed after 2 weeks.

Subcutaneous tumorigenicity assay

For the tumorigenicity assays, eight-week-old immunocompromised athymic nude mice (Hsd:Athymic nude-nu, Harlan) were used. Cells (5×10^6) were resuspended in 100 μl of PBS and injected with a 25 gauge needle into anaesthetized mice.

BJ cells infected with the E1A/ras combination were also injected into 10 nude mice γ-irradiated with 400 rad prior injection and into 6 SCID beige mice (C.B-17/IcrHsd-scid-bg, Harlan). Tumor size was monitored every 5 days. Mice were sacrificed when the tumors reached a diameter of 1–1.2 cm or after 16 weeks of monitoring.

Tumors were collected in a sterile field and minced. Tumor fragments were immediately frozen in liquid nitrogen for DNA and protein extraction and for telomerase assays. Other fragments were fixed in 10% formalin for histological and immunohistochemical examinations. Finally, fragments were finely minced, washed in PBS, and plated in culture medium for isolation of tumor cells.

Tumor morphological and histochemical examination

Formalin-fixed/paraffin-embedded or snap-frozen fragments of tumor specimens were stained with hematoxylin and eosin and with histochemical stains (PAS and PAS after diastase, Reticulum, and Masson's trichrome stain) for morphological evaluation and histochemical analyses. Immunohistochemistry was performed with the peroxidase ARK kit (DAKO Glostrup Denmark) and DAB as chromogen. The following primary antibodies were utilized: Intermediate filaments (Vimentin, pan-keratin, desmin) and other human specific monoclonal antibodies (S100 protein, EMA, CD45) were used as histogenetic markers. The expression of ectopically expressed oncogenes was determined by using monoclonal antibodies specific for MDM2 (4B2) (a kind gift from A. Levine), p21-ras (OP23) (Oncogene Research Products), and E1A (M73).

Scoring of chromosomal abnormalities

Karyotype analysis

Metaphase chromosomes preparation from explanted tumor cells and quinacrine banding (QFQ staining) were according to standard protocols.

Q-FISH

The indicated numbers of metaphases from each culture were scored for chromosomal aberrations by superimposing the telomere image on the DAPI chromosome image in the TFL-telo software (gift from Dr. Peter Lansdorp, Vancouver). End-to-end fusions can be two chromosomes fused by their p-arms (Robertsonian-like fusions) or two chromosomes fused by their q-arms (dicentrics).

Clonality analysis

To confirm the polyclonality of tumor cell population, genomic DNA was extracted from parental and explanted tumor cells by conventional Proteinase K/SDS digestion. Twelve micrograms of DNA were digested with either EcoRI or BamHI, alone or in combination with XhoI or SalI, and fractionated in a 0.8% agarose gel. After transfer onto Hybond N+ membrane (Amersham), blots were hybridized with ^{32}P-labeled probes specific for mdm2, E1A, or Ha-ras. Membranes were hybridized overnight at 65°C in 0.2 M $NaPO_4$, 1 mM EDTA, 7% SDS, 1% BSA in the presence of 15% formamide. Membranes were washed twice in 0.1% SDS, 0.2× SSC and once in 0.1× SSC at 60°C, followed by autoradiography.

Telomere length measurements

Q-FISH on metaphasic chromosomes

Metaphases were prepared for Q-FISH and hybridized as described (Samper et al., 2000, 2001). To correct for lamp intensity and alignment, images from fluorescent beads (Molecular probes, USA) were analyzed using the TFL-Telo program. Telomere fluorescence values were extrapolated from the telomere fluorescence of LY-R (R cells) and LY-S (S cells) lymphoma cell lines of known lengths of 80 and 10 kb (McIlrath et al. 2001). There was a linear correlation (r2 = 0.999) between the fluorescence intensity of the R and S telomeres with a slope of 38.6. The calibration-corrected telomere fluorescence intensity (ccTFI) was calculated as described (Herrera et al., 1999).

Images were captured using Leica Q-FISH software at 400 mSec integration-time in a linear acquisition mode to prevent oversaturation of fluorescence intensity and recorded using a COHU CCD camera on a Leica Leitz DMRB fluorescence microscope.

TFL-Telo software (gift from Dr. Lansdorp, Vancouver) was used to quantify the fluorescence intensity of telomeres from at least 10 metaphases of each data point. The images of metaphases from different cell cultures were captured on the same day in parallel, and scored blind.

Terminal restriction fragment analysis (TRF)

Cells were prepared in agarose plugs and digested with Mbo I for TRF analysis using pulse-field electrophoresis as described in Blasco et al. (1997b).

Telomerase assays

Telomerase activity was measured with a modified telomeric repeat amplification protocol (TRAP), as described (Blasco et al., 1997b).

Analysis of hTERT mRNA expression by RT-PCR

Analysis of human TERT expression was carried out by RT-PCR. cDNA was synthesized from 1 μg of total RNA using random primers in a 20 μl reaction. 1 μl of cDNA was then used to amplify two fragments of human TERT sequence, both spanning an intronic sequence. Primers hTERT1s, 5′-TTCCT GCACTGGCTGATGAGTGT-3′ and hTERT1a, 5′-AGCGTCGGCCCTCTTTTC TCTG-3′ were used to amplify a 330 bp fragment spanning exons 3 and 4 of the human TERT sequence. Primers hTERT2s, 5′-ACAGCACTTGAA GAGGGTG-3′ and hTERT2a, 5′-GTGCCTTCACCCTCGAGG-3′ were used to amplify a 210 bp fragment spanning exons 4 and 5 of the human TERT sequence. Both PCR reactions were carried out for 40 or 50 cycles with an elongation at 72°C for 30 s and annealing at 65°C. PCR products were analyzed on a 3% agarose gel. The quality of cDNA was controlled by PCR amplification of a 500 bp fragment of a β-actin transcript in a 20 cycle-PCR reaction.

Acknowledgments

We thank Scott Lowe and Andrew Samuelson for their kind gifts of reagents and for helpful discussions. We also thank Silvia Demontis and Michela Armellin for helpful suggestions and support. Y.R.S. is supported by a predoctoral fellowship from the U.S. Army Breast Cancer Research Program (DAMD 17-01-1-0210). M.G-C. is a predoctoral student of the Spanish Ministry of Science and Technology. Research at the laboratory of M.A.B. is funded by grants PM97-0133 from the MCYT, 08.1/0030/98 from CAM, and by grants EURATOM/991/0201, FIGH-CT-1999-00002, and FIS5-1999-00055 from the European Union, and by the DIO. The DIO was founded and is supported by the Spanish Research Council (CSIC) and by Pharmacia. R.M. and C.D. are supported by grants from the Italian Association for Cancer Research (AIRC). G.J.H. is supported by an Innovator Award from the U.S. Army Breast Cancer Research program, is a Rita Allen Scholar, and is supported by grants from the N.C.I. (2P01-CA13106) and from the U.S. Army Breast Cancer Research Program (DAMD-17-0010-207, DAMD-17-0010-208, DAMD-17-02-1-0346).

Received: May 28, 2002
Revised: September 16, 2002

References

Albanell, J., Lonardo, F., Rusch, V., Engelhardt, M., Langenfeld, J., Han, W., Klimstra, D., Venkatraman, E., Moore, M.A., and Dmitrovsky, E. (1997). High telomerase activity in primary lung cancers: association with increased cell proliferation rates and advanced pathologic stage. J. Natl. Cancer Inst. *89*, 1609–1615.

Barbacid, M. (1987). Ras genes. Annu. Rev. Biochem. *56*, 779–827.

Barbeau, D., Charbonneau, R., Whalen, S.G., Bayley, S.T., and Branton, P.E. (1994). Functional interactions within adenovirus E1A protein complexes. Oncogene *9*, 359–373.

Blasco, M.A., Lee, H.W., Rizen, M., Hanahan, D., DePinho, R., and Greider,

C.W. (1997a). Mouse models for the study of telomerase. Ciba Found. Symp. *211*, 160–176.

Blasco, M.A., Lee, H.W., Hande, M.P., Samper, E., Lansdorp, P.M., DePinho, R.A., and Greider, C.W. (1997b). Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell *91*, 25–34.

Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S., and Wright, W.E. (1998). Extension of life-span by introduction of telomerase into normal human cells. Science *279*, 349–352.

Boyd, J.M., Subramanian, T., Schaeper, U., La Regina, M., Bayley, S., and Chinnadurai, D. (1993). A region in the C-terminus of adenovirus 2/5 E1A protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis, and metastasis. EMBO J. *12*, 469–478.

Bryan, T.M., and Reddel, R.R. (1997). Telomere dynamics and telomerase activity in *in vitro* immortalised cells. Eur. J. Cancer *33*, 767–773.

Bryan, T.M., Englezou, A., Dalla-Pozza, L., Dunham, M.A., and Reddel, R.R. (1997). Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat. Med. *3*, 1271–1274.

Chadeneau, C., Hay, K., Hirte, H.W., Gallinger, S., and Bacchetti, S. (1995). Telomerase activity associated with acquisition of malignancy in human colorectal cancer. Cancer Res. *55*, 2533–2536.

Chin, L., Artandi, S.E., Shen, Q., Tam, A., Lee, S.L., Gottleib, G.J., Greider, C.W., and DePinho, R.A. (1999). p53-deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. Cell *97*, 527–538.

Counter, C.M., Hahn, W.C., Wei, W., Caddle, S.D., Beijersbergen, R.L., Lansdorp, P.M., Sedivy, J.M., and Weinberg, R.A. (1998). Dissociation among in vitro telomerase activity, telomere maintenance, and cellular immortalization. Proc. Natl. Acad. Sci. USA *95*, 14723–14728.

Debbas, M., and White, E. (1993). Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. *7*, 546–554.

Dei Tos, A.P., Piccinin, S., Doglioni, C., Vukosavljevic, T., Mentzel, T., Boiocchi, M., and Fletcher, C.D. (1997). Molecular aberrations of the G1-S checkpoint in myxoid and round cell liposarcoma. Am. J. Pathol. *151*, 1531–1539.

de Stanchina, E., McCurrach, M.E., Zindy, F., Shieh, S.Y., Ferbeyre, G., Samuelson, A.V., Roussel, M.F., Sherr, C.J., and Lowe, S.W. (1998). E1A signaling to p53 involves the p19(ARF) tumor suppressor. Genes Dev. *12*, 2434–2442.

Dorsman, J.C., Hagmeyer, B.M., Veenstra, J., Elfferich, N.N., Zantema, A., and Van Der Eb, A.J. (1995). The N-terminal region of the adenovirus type 5 E1A proteins can repress expression of cellular genes via two distinct but overlapping domains. J. Virol. *69*, 2962–2967.

Dunham, M.A., Neumann, A.A., Fasching, C.L., and Reddel, R.R. (2000). Telomere maintenance by recombinantion in human cells. Nat. Genet. *26*, 447–450.

Ebina, Y., Yamada, H., Fujino, T., Furuta, I., Sakuragi, N., Yamamoto, R., Katoh, M., Oshimura, M., and Fujimoto, S. (1999). Telomerase activity correlates with histo-pathological factors in uterine endometrial carcinoma. Int. J. Cancer *84*, 529–532.

Elenbaas, B., Spirio, L., Koerner, F., Fleming, M.D., Zimonjic, D.B., Donaher, J.L., Popescu, N.C., Hahn, W.C., and Weinberg, R.A. (2001). Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev. *15*, 50–65.

Feuer, G., Stewart, S.A., Baird, S.M., Lee, F., Feuer, R., and Chen, I.S. (1995). Potential role of natural killer cells in controlling tumorigenesis by human T-cell leukemia viruses. J. Virol. *69*, 1328–1333.

Fuchs, M., Gerber, J., Drapkin, R., Sif, S., Ikura, T., Ogryzko, V., Lane, W.S., Nakatani, Y., and Livingston, D.M. (2001). The p400 complex is an essential E1A transformation target. Cell *106*, 297–307.

González-Suárez, E., Samper, E., Flores, J.M., and Blasco, M.A. (2000). Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis. Nat. Genet. *26*, 114–117.

Goodman, R.H., and Smolik, S. (2000). CBP/p300 in cell growth, transformation, and development. Genes Dev. *14*, 1553–1577.

Greenberg, R.A., Chin, L., Femino, A., Lee, K.H., Gottlieb, G.J., Singer, R.H., Greider, C.W., and DePinho, R.A. (1999). Short dysfunctional telomeres impair tumorigenesis in the INK4a(delta2/3) cancer-prone mouse. Cell *97*, 515–525.

Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, R.A. (1999). Creation of human tumor cells with defined genetic elements. Nature *400*, 464–468.

Hahn, W.C., Dessain, S.K., Brooks, M.W., King, J.E., Elenbaas, B., Sabatini, D.M., DeCaprio, J.A., and Weinberg, R.A. (2002). Enumeration of the simian virus 40 early region elements necessary for human cell transformation. Mol. Cell. Biol. *22*, 2111–2123.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57–70.

Hannon, G.J., Sun, P., Carnero, A., Xie, L.Y., Maestro, R., Conklin, D.S., and Beach, D. (1999). MaRX: an approach to genetics in mammalian cells. Science *283*, 1129–1130.

Harlow, E., Whyte, P., Franza, B.R., Jr., and Schley, C. (1986). Association of adenovirus early-region 1A proteins with cellular polypeptides. Mol. Cell. Biol. *6*, 1579–1589.

Harlow, E., and Lane, D. (1999). Using Antibodies: A Laboratory Manual. (Cold Spring Harbor: Cold Spring Harbor Laboratory Press).

Harrington, E.A., Bennett, M.R., Fanindi, A., and Evan, G.I. (1994). c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. EMBO J. *13*, 3286–3295.

Hermeking, H., and Eick, D. (1994). Mediation of c-Myc-induced apoptosis by p53. Science *265*, 2091–2093.

Herrera, E., Samper, E., Martin-Caballero, J., Flores, J.M., Lee, H.W., and Blasco, M.A. (1999). Disease states associated with telomerase deficiency appear earlier in mice with short telomeres. EMBO J. *18*, 2950–2960.

Herrera, E., Martinez, A.C., and Blasco, M.A. (2000). Impaired germinal center reaction in mice with short telomeres. EMBO J. *19*, 472–481.

Hiyama, E., Hiyama, K., Yokoyama, T., Matsuura, Y., Piatyszek, M.A., and Shay, J.W. (1995). Correlating telomerase activity levels with human neuroblastoma outcomes. Nat. Med. *1*, 249–255.

Hoare, S.F., Bryce, L.A., Wisman, G.B., Burns, S., Going, J.J., van der Zee, A.G., and Keith, W.N. (2001). Lack of telomerase RNA gene hTERC expression in alternative lengthening of telomeres cells is associated with methylation of the hTERC promoter. Cancer Res. *61*, 27–32.

Imam, S.A., Kim, M.S., Anker, L., Datar, R.H., Law, R.E., and Taylor, C.R. (1997). Systematic determination of telomerase activity and telomere length during the progression of human breast cancer in cell culture models. Anticancer Res. *17*, 4435–4441.

Kamijo, T., Zindy, F., Roussel, M.F., Quelle, D.E., Downing, J.R., Ashmun, R.A., Grosveld, G., and Sherr, C.J. (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell *91*, 649–659.

Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L., Coviello, G.M., Wright, W.E., Weinrich, S.L., and Shay, J.W. (1994). Specific association of human telomerase activity with immortal cells and cancer. Science *266*, 2011–2015.

Land, H., Parada, L.F., and Weinberg, R.A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature *304*, 596–602.

Lee, H.W., Blasco, M.A., Gottliebb, G.J., Horner, J.W., 2nd, Greider, C.W., and DePinho, R.A. (1998). Essential role of mouse telomerase in highly proliferative organs. Nature *392*, 569–574.

Lin, A.W., Barradas, M., Stone, J.C., van Aelst, L., Serrano, M., and Lowe, S.W. (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 Ruley, H.E. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev. *7*, 535–545.

Lowe, S.W., Bodis, S., McClatchey, A., Remington, L., Ruley, H.E., Fisher, D.E., Housman, D.E., and Jacks, T. (1994). p53 status and the efficacy of cancer therapy in vivo. Science *266*, 807–810.

McEachern, M.J., Krauskopf, A., and Blackburn, E.H. (2000). Telomeres and their control. Annu. Rev. Genet. *34*, 331–358.

McIlrath, J., Bouffler, S.D., Samper, E., Cuthbert, A., Wojcik, A., Szumiel, I., Bryant, P.E., Riches, A.C., Thompson, A., Blasco, M.A., et al. (2001). Telomere length abnormalities in mammalian radiosensitive cells. Cancer Res. *61*, 912–915.

McMahon, S.B., Van Buskirk, H.A., Dugan, K.A., Copeland, T.D., and Cole, M.D. (1998). The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell *94*, 363–374.

Meloni, A.R., Smith, E.J., and Nevins, J.R. (1999). A mechanism for Rb/p130-mediated transcription repression involving recruitment of the CtBP corepressor. Proc. Natl. Acad. Sci. USA *96*, 9574–9579.

Meyerson, M., Counter, C.M., Eaton, E.N., Ellisen, L.W., Steiner, P., Caddle, S.D., Ziaugra, L., Beijersbergen, R.L., Davidoff, M.J., Liu, Q., et al. (1997). hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells during immortalization. Cell *90*, 785–795.

Nanda, I., Schneider-Rasp, S., Winking, H., and Schmid, M. (1995). Loss of telomeric sites in the chromosomes of mus musculus domesticus (Rodentia: Muridae) during Robertsonian rearrangements. Chromosome Res. *3*, 399–409.

O'Brien, W., Stenman, G., and Sager, R. (1986). Suppression of tumor growth by senescence in virally transformed human fibroblasts. Proc. Natl. Acad. Sci. USA *83*, 8659–8663.

Paulovich, A.G., Toczyski, D.P., and Hartwell, L.H. (1997). When checkpoints fail. Cell *88*, 315–322.

Rudolph, K.L., Millard, M., Bosenberg, M.W., and DePinho, R.A. (2001). Telomere dysfunction and evolution of intestinal carcinoma in mice and humans. Nat. Genet. *28*, 155–159.

Ruley, H.E. (1983). Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature *304*, 602–606.

Sager, R. (1991). Senescence as a mode of tumor suppression. Environ. Health Perspect. *93*, 59–62.

Samper, E., Goytisolo, F.A., Slijepcevic, P., van Buul, P.P., and Blasco, M.A. (2000). Mammalian Ku86 protein prevents telomeric fusions independently of the length of TTAGGG repeats and the G-strand overhang. EMBO Rep. *1*, 244–252.

Samper, E., Goytisolo, F.A., Menissier-de Murcia, J., Gonzalez-Suarez, E., Cigudosa, J.C., de Murcia, G., and Blasco, M.A. (2001). Normal telomere length and chromosomal end capping in poly (ADP-ribose) polymerase-deficient mice and primary cells despite increased chromosomal instability. J. Cell Biol. *154*, 49–60.

Samuelson, A.V., and Lowe, S.W. (1997). Selective induction of p53 and chemosensitivity in RB-deficient cells by E1A mutants unable to bind the RB-related proteins. Proc. Natl. Acad. Sci. USA *94*, 12094–12099.

Sherr, C.J. (1996). Cancer cell cycles. Science *274*, 1672–1677.

Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R.A. (1996). Role of the INK4a locus in tumor suppression and cell mortality. Cell *85*, 27–37.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D.H., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593–602.

Stevenson, M., and Volsky, D.J. (1986). Activated v-myc and v-ras oncogenes do not transform normal human lymphocytes. Mol. Cell. Biol. *6*, 3410–3417.

Tang, R., Cheng, A.J., Wang, J.Y., and Wang, T.C. (1998). Close correlation between telomerase expression and adenomatous polyp progression in multistep colorectal carcinogenesis. Cancer Res. *58*, 4052–4054.

Wang, H.H., Moran, E., and Yaciuk, P. (1995). E1A promotes association between p300 and pRb in multimeric complexes required for normal biological activity. J. Virol. *69*, 7917–7924.

Wang, J., Xie, L.Y., Allan, S., Beach, D., and Hannon, G.J. (1998). Myc activates telomerase. Genes Dev. *12*, 1769–1774.

Webb, C.P., Van Aelst, L., Wigler, M.H., and Woude, G.F. (1998). Signaling pathways in Ras-mediated tumorigenicity and metastasis. Proc. Natl. Acad. Sci. USA *95*, 8773–8778.

Whyte, P., Buchkovich, K.J., Horowitz, J.M., Friend, S.H., Raybuck, M., Weinberg, R.A., and Harlow, E. (1988). Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature *334*, 124–129.

Whyte, P., Williamson, N.M., and Harlow, E. (1989). Cellular targets for transformation by the adenovirus E1A proteins. Cell *56*, 67–75.

Wright, W.E., Shay, J.W., and Piatyszek, M.A. (1995). Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity, and sensitivity. Nucleic Acids Res. *23*, 3794–3795.

Yan, P., Coindre, J.M., Benhattar, J., Bosman, F.T., and Guillou, L. (1999). Telomerase activity and human telomerase reverse transcriptase mRNA expression in soft tissue tumors: correlation with grade, histology, and proliferative activity. Cancer Res. *59*, 3166–3170.