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Award Number:  DAMD17-00-1-0208

TITLE:  Synthetic Lethality in Breast Cancer Cells:  Genes
Required for Tumor Survival

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REPORT DATE:  June 2001

TYPE OF REPORT:  Annual

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

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**20011005 305**

| REPORT DOCUMENTATION PAGE | | *Form Approved* <br> *OMB No. 074-0188* |
|---|---|---|

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| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE <br> June 2001 | 3. REPORT TYPE AND DATES COVERED <br> Annual    (1 Jun 00 – 31 May 01) | |
|---|---|---|---|

**4. TITLE AND SUBTITLE**

Synthetic Lethality in Breast Cancer Cells:  Genes Required for Tumor Survival

**5. FUNDING NUMBERS**
DAMD17-00-1-0208

**6. AUTHOR(S)**
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**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**
Report contains color

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

**12b. DISTRIBUTION CODE**

**13. Abstract** *(Maximum 200 Words)* (abstract should contain no proprietary or confidential information)
The conversion of a normal cell into a cancer cell proceeds through a series of genetic and epigenetic alterations.  We have proposed to use well-established genetic methodologies to identify novel anti-cancer targets via their specific, genetic interactions with common cancer mutations.  In short, we will identify genetic alterations that are neutral in normal cells, but that are lethal when combined with cancer mutations. This "synthetic lethality" approach may identify potential therapeutic targets that are highly specific to the cancer cell.  In the past year, we have made substantial progress toward the goal of developing technologies necessary to making this type of target search a reality.  We have devised genetically defined human cancer models that can be used to conduct synthetic lethality screens and have developed new methodologies for manipulating gene expression in mammalian cells.  This work has resulting in two manuscripts that are now under review for publication.

**14. SUBJECT TERMS**
cancer biology, genetics, synthetic lethality, apoptosis

**15. NUMBER OF PAGES**
74

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT <br> Unclassified | 18. SECURITY CLASSIFICATION OF THIS PAGE <br> Unclassified | 19. SECURITY CLASSIFICATION OF ABSTRACT <br> Unclassified | 20. LIMITATION OF ABSTRACT <br> Unlimited |
|---|---|---|---|

NSN 7540-01-280-5500

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

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# Introduction

The original goals of this proposal were to develop methodologies that will permit the identification of new anticancer targets via a genetic approach. Using the well-established genetic principle of synthetic lethality, we hoped to find genetic alterations that would not be tolerated when combined with common oncogene and tumor suppressor mutations. We have examined a number of the strategies proposed in the original application and feel that most do not give sufficient sensitivity to permit genetic selection. We have therefore been forced to rethink our approach. While the overall goal of the grant is unchanged, we feel that we now have a much more workable strategy for identifying synthetic lethal interactions in mammalian cells, and plan to devote the remainder of our efforts to making this work. Our shift in this direction has hinged upon two things. First, we realized that we needed defined models of human cancer cells in order to make synthetic lethal screens workable. Through substantial efforts, we have developed combinations of viral and cellular oncogenes that can be used to transform normal human cells, at will. These will be used as a manipulatable context for our genetic screens. Second, it was clearly essential to devise better methodologies for suppressing gene expression in mammalian cells. Toward this goal, we have been working for some years on the mechanistic basis of RNAi. We have recently shown that RNAi can be used to suppress gene expression in mammalian cells. We now feel that the combination of these approaches can be applied to the identification of novel anticancer targets via synthetic lethality screens.

**Body**

*1. Genetically defined models for human cell transformation*

One route toward the identification of novel anti-cancer targets is via a detailed understanding of the pathways that lead to tumorigenesis. We argued in the original proposal, and continue to believe, that it is better to take an unbased, genetic approach toward the problem. However, we have recognized that in order to permit genetics to be applied to a search for novel therapeutic targets, we require a defined cell system. For this reason, we have striven, over the past several years, to identify a combination of viral and cellular oncogenes that could transform normal human cells into cancer cells. This work is described in detail in an appended manuscript (Seger et al.; Appendix 1) that has been submitted for review.

Our founding observation was that combined expression of adenovirus E1A and activated, Ha-rasV12 was sufficient to permit anchorage-independent growth of normal human fibroblasts. This combination was, however, insufficient to promote tumor formation in mice. Over the course of our studies, we noted that out of ~100 injections of E1A/ras cells, one tumor did form after a long latency period. We reasoned that this tumor resulted from that acquisition of additional genetic alterations, and perhaps only one additional alteration. We therefore searched for an oncogene that would permit tumor formation when combined with E1A and ras. Based upon prior studies in mouse cells, we tested whether negation of the p53 pathway was that additional hit. Indeed, combined expression of E1A, Ha-rasV12

and mdm2 (ERM) was sufficient to convert a normal human cell into a cancer cell. These cells form tumors in ~80% of injections into immunocompromised mice.

In the two previous reports of human cell transformation from the Weinberg group, activation of telomerase and cell immortalization was essential for transformation. However, we had previously shown that none of the three oncogenes in our transformation model could activate telomerase, on its own. Consistent with our prior observations, ERM cells are telomerase negative prior to injection into mice and give rise to telomerase-negative tumors. Cells explanted from those tumors show chromosome abnormalities that are consistent with telomere loss in ~100% of metaphases (end-to-end fusions, ring chromosomes). Thus, we have shown that activation of telomerase is not obligate for the conversion of normal human cells into cancer cells.

Using pre-existing and well-characterized E1A mutants, we have begun to examine the cellular pathways that must be targeted by E1A to effect human cell transformation. Thus far, we have demonstrated that the c-terminus of the protein, which binds to CtBP, is not required however, binding to p300 and the Rb family is required. In addition, we have shown that a region spanning residues 26-35 is essential. This region has recently been shown to bind to a complex that contains p400 and the myc-binding protein, TRRAP. Indeed ectopic expression of c-myc will complement the Δ26-35 mutant (but not other mutants) in human cell transformation, providing the first model in which we can examine the roles played by myc in a defined model of human cancer.

We have recently tested whether enforced, mdm2 expression could be substituted by loss of p53 (via expression of dominant negative mutants) or by inhibition of apoptosis (via overexpression BCL2). Surprisingly, neither of these could complement mdm2, strongly suggesting that mdm2 not only has functions in addition to its ability to inhibit p53 function (and this has been previously suggested by a growing body of literature) but also that those functions could be read-out in our transformation model. We are currently pursuing these p53-independent mdm2 pathways using genetic approaches (mutations, complementation etc) and microarrays.

It is now possible to use these cells in a screen for genetic alterations that are synthetically lethal with changes in each of the cancer pathways that are affected in our transformation model.

## 2. Loss-of-function Genetics – RNAi in mammalian cells

Our continuing work with antisense RNA has made it obvious that for synthetic lethal screens to work, we needed a better loss-of-function tool. For this reason, we turned, a number of years ago, to studies on RNAi with the intension of trying ultimately to use this approach to manipulate gene expression in mammalian cells. In short, we and others have recently succeeded in demonstrating that RNAi pathways exist and are functional in some mammalian cell types. This work is described in detail in the attached manuscript that is presently being revised for re-submission (see Paddison et al., Appendix 2).

*3. Overview*

We now feel that the combination of genetically defined cell models for transformation and the ability to manipulate gene expression via RNAi in mammals gives us the combination of tools necessary to proceed with synthetic lethal screens. Of course, we still have additional technological barriers that must be overcome to achieve this goal. Making stable RNAi in mammalian cells and creating RNAi libraries are two such hurdles that will form the core of our work in year 2 of the grant. However, considering the progress that we have made thus far, these barriers seem surmountable.

**Key Research accomplishments**

- Development of a genetically defined system for human cell transformation

- Demonstration that transformation proceeds without telomerase activation

- Mapping cellular pathways that are required for human cell transformation

- Demonstration that RNAi can be used to suppress gene function in mammalian cells

**Reportable outcomes**

- Two manuscripts that are presently being reviewed are attached

- Cells that have been transformed via the combination of E1A/Ha-rasV12/MDM2

- This grant supported the development of the transformation system, analysis of which has become the subject of a section of a P01 grant that is likely to be funded

## Conclusions

The identification of novel anticancer targets is a principal goal of the war on cancer. We have for some years hoped to take a genetic approach to this problem. During the past year, we have made two major steps toward enabling this approach. First, we have developed genetically defined transformation models. Second, we have developed new loss-of-function methodologies that can be applied to human cancer cells. During the coming year, we hope to build upon these accomplishments to devise a screen for synthetic lethal interactions that can be carried out during the third year of support.

Transformation of normal human cells by combined expression of E1A, Ha-rasV12 and MDM2 in the absence of telomerase activation

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Running title : Human cell transformation

Key Words : adenovirus E1A; Ha-rasV12; MDM2; Telomerase; transformation

**Abstract**

Normal cells become transformed into tumor cells via cooperating genetic and epigenetic events, which result in the activation of oncogenes and the inactivation of tumor suppressors.  Much of what is known of the transformation process has emerged from studies of primary rodent cells and animals.  However, it is clear that these models do not perfectly recapitulate the transformation process in humans.  In fact, it is impossible to transform normal human cells into tumor cells using the same combinations of oncogenic alterations that are effective in rodent cells.  Here, we report that combined expression of adenovirus E1A, activated Ha-rasV12 and MDM2 is sufficient to convert a normal human cell into a cancer cell.  Through analysis of E1A deletion mutants, we begin to map the cellular pathways, which must be altered to elicit transformation.  Notably, transformation by cooperating oncogenes occurred without telomerase activation.  This suggests that telomere maintenance is not an obligate characteristic of tumorigenic human cells.

**Introduction**

Neoplastic transformation occurs via a series of genetic and epigenetic alterations, which result in a cell population that is capable of proliferating independently of both external and internal signals that normally restrain growth.  For example, transformed cells show a reduced requirement for extracellular

growth promoting factors, are not restricted by signals that result from normal cell-cell contact, and are often immortal (Paulovich et al., 1997; Hanahan and Weinberg, 2000). Through extensive studies of transformation processes in rodent cell 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 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, indicating fundamental differences in requirements for transformation in human versus rodent cells (Blasco et al., 1997a; Holt and Shay, 1999).

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 effect tumor suppression. For example, activation of oncogenes such c-Myc or adenovirus E1A sensitize primary cells to apoptosis (Debbas and White, 1993; Lowe et al., 1994; Lowe and Ruley, 1993; Harrington et al., 1994; Hermeking and Eick, 1994; Wagner et al., 1994). Hyper-activation 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 p19ARF can be transformed directly by activated ras (Kamijo et al., 1997; Serrano et al., 1996; Serrano et al., 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 utilize multiple, independent and redundant effector pathways.

A second characteristic that distinguishes primary human and murine cells is that the latter are easily immortalized (Blasco et al., 1997). 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 *. The potential importance of telomerase in human tumorigenesis models is supported by numerous observations. First, the majority of human tumors are telomerase-positive *(kim). 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). Here we report an alternative model of human cell transformation. We show that co-expression of two oncogenes, adenovirus E1A and Ha-RasV12 is sufficient to enable primary human fibroblasts to grow in the absence of anchorage, a hallmark of transformation *in vitro*. However, this combination is insufficient to permit tumor formation in nude mice. Addition of a third oncogene, MDM2, can convert these into cells capable of forming tumors *in vivo*. Of interest, 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 in human cells.

**Results**

*Co-expression of E1A and Ha-RasV12 in normal human fibroblasts results in 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 murine cells, *c-myc* is capable of bypassing ras-induced growth arrest and of cooperating with activated, *Ha-rasV12* to transform primary rodent cells into tumorigenic cells (Land et al., 1983). However, combined expression of myc and activated ras in normal human cells not only fails to transform but also leads to accelerated appearance of the senescent-like phenotype (Fig 1A, and data not shown).

Whereas numerous genetic alterations have been shown to bypass ras-induced growth arrest in murine cells, only very few have a demonstrated capability of overriding this response in normal human cells. One of these is ectopic expression of the adenovirus oncogene, E1A (Fig. 1A) (Serrano et al., 1997; de Stanchina et al., 1998). Indeed, coordinate expression of *E1A* and *Ha-rasV12* provided one of the first demonstrations that cooperating oncogenes that could transform normal rodent cells (Ruley, 1983). We, therefore, tested whether combined expression of E1A and Ha-rasV12 could transform normal human fibroblasts.

A characteristic feature of transformed cells is their ability to grow in the absence of anchorage and, therefore, to form colonies in semisolid media. BJ fibroblasts or cells expressing E1A or Ha-RasV12, individually, failed to form

colonies in soft agar. However, cells expressing both E1A and Ha-RasV12

formed colonies in soft agar with an efficiency that is comparable to that seen

with transformed human and rodent cells (Fig. 1B). For human 293 cells,

virtually all plated cells gave rise to colonies compared to 20-60% for BJ/E1A/Ha-

RasV12 (depending upon the precise combination of expression vectors, Fig.

1C). In general, colonies generated by BJ/ER (E=E1A, R=Ha-rasV12) contain

fewer cells than those generated by 293 cells within the same time period.

BJ fibroblasts coexpressing E1A and Ha-RasV12 were next 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 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, non-irradiated mouse (Table 1). This tumor arose after a substantially

longer latency (10 weeks) than is normally observed using control cancer cell

lines or transformed, human 293 cells (~2 weeks), suggesting the possibility that

an additional, rare genetic alteration may have contributed to tumor formation in

this individual case. Thus, the combination of E1A and Ha-RasV12, although

sufficient to permit anchorage-independent growth of normal human fibroblasts,

is insufficient to permit tumor formation in nude mice.

*Co-expression of 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 indicated that these oncogenes transformed much more efficiently in the absence of p53 (Lowe et al., 1993). Indeed tumors generally arose from E1A/ras transformed MEF only after a long latency period, and most of these lacked a functional p53 pathway. Interestingly, immunohistochemical analysis revealed that the single tumor that was produced by the E1A/Ha-RasV12-expressing cells showed a strong accumulation of nuclear p53, but SSCP analysis excluded the presence of p53 gene mutations (data not shown). Accumulation of wild type p53 is a common feature of human sarcomas, the type of tumors that arise from the same precursors that differentiate into fibroblasts. These tumors often show overexpression of MDM2 (Dei Tos et al., 1997), indicating that negation of p53 function often occurs in sarcomas through mechanisms other than p53 gene mutation. Furthermore, the tumor formed from the E1A/Ha-rasV12-expressing fibroblasts was negative for the expression of ARF, an upstream regulator of MDM2, whereas the pre-injection population of engineered fibroblasts expressed ARF abundantly (as do tumors formed from the engineered fibroblasts discussed below). We therefore 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 were simultaneously co-infected with three retroviruses that direct the expression of E1A, MDM2, and Ha-RasV12, with each retrovirus bearing a different drug selection marker. Control cells were prepared replacing individual oncogene-expressing viruses with an empty vector bearing the same selection

marker.  These triply-infected populations were simultaneously co-selected for puromycin, hygromycin, and neomycin resistance for seven days and were then plated into soft agar or injected into immunocompromised mice.  Cell populations expressing E1A/Ha-RasV12 or E1A/MDM2/Ha-RasV12 both formed colonies in soft agar with comparable efficiency (Figs. 1B, 2AB). However, only the triply-infected cells formed tumors in mice (Figs 2CD, Table 1).  Tumors grew to a size at which the animals had to be sacrificed within a period of from three to six weeks, a latency comparable to that seen with control human cancer cell lines or with transformed 293T cells (Fig 2C,D).

In order to determine whether rare, additional genetic events had been selected during the *in vivo* tumorigenesis assay, retroviral integration sites were analyzed by Southern blotting with probes to drug resistance markers.  This indicated that cell populations remained polyclonal throughout drug selection *in vitro* and tumorigenesis *in vivo* (not shown).  These results argue against selection for rare genetic events during tumor formation, and suggest the possibility that the combined expression of E1A, MDM2, and Ha-RasV12 is sufficient for the transformation of normal human fibroblasts into tumor cells.

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

Cell immortalization has been posited as a landmark event in the progression form a normal cell into a cancer cell.  Indeed, most human cancers are positive for telomerase, an indirect indication that these cells have evolved

mechanisms for telomere maintenance and extension of proliferative capacity *(kim). In previous reports, transformation of normal human cells absolutely required activation of telomerase via expression of its limiting, catalytic subunit, hTERT (Hahn et al., 1999; Elenbaas et al., 2001). We previously showed that E1A, Ha-rasV12 and MDM2 were each 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 without telomerase activation.

Telomerase activity was easily detectable telomerase activity in 293 cells. Based upon serial dilutions, as few as 10 293 cells yielded a strong, positive signal in our assays (not shown). As expected, BJ fibroblasts are telomerase-negative. We similarly fail to detect telomerase in BJ cells that have been engineered to express E1A, Ha-rasV12 and MDM2 (ERM, Fig. 3A). Based upon mixing experiments, we conclude that BJ/ERM cells are telomerase-negative, or contain at least 1000-fold less telomerase activity than do 293 cells. Furthermore, telomerase is undetectable in tumors that form following injection of *in vitro* transformed BJ/ERM fibroblasts into mice (Fig. 3B).

Immortal cells can maintain telomere length through at least two independent mechanisms. By far the most common is telomerase activation; however, alternative pathways of telomere maintenance (ALT) have also been

described *(r. reddell papers). Thus, it was possible that BJ-derived tumor cells had evolved mechanisms of telomere maintenance that were not reflected in telomerase activity. We therefore analyzed telomeric restriction fragment lengths (Harley et al., 1990) in BJ cells prior to infection, in BJ cells that were engineered to express E1A/Ha-rasV12 and MDM2 and passaged in vitro, and in tumors that formed upon injection of engineered BJ cells. In all cases, a continuous erosion of telomere length was evident and correlated with the proliferation of these cells *in vitro* or *in vivo* (not shown).

As noted above, BJ cells are engineered to express E1A, Ha-rasV12 and MDM2 by simultaneous co-infection. It is, therefore, not surprising to find that the karyotype of the engineered cells is normal prior to injection into mice, since these cells have not undergone prolonged expansion in the presence of any individual oncogene (Fig. 3C). However, examination of cells that are explanted following tumor formation reveals numerous genetic abnormalities (Fig. 3D). In virtually every metaphase, we noted the presence of dicentric chromosomes that apparently formed via end-to-end fusion. In some metaphases, we also find ring chromosomes (Fig. 3D). These types of genetic abnormalities are a characteristic outcome of telomere depletion (Blasco et al., 1997b; Nanda et al., 1995). Considered together, the results of telomerase enzyme assays, of telomeric restriction fragment analysis and of cytogenetic examination of explanted tumor cells strongly suggest that combined expression of E1A, Ha-rasV12 and MDM2 is capable of transforming normal cells into human tumor

cells without activation of telomerase or alternative mechanisms of telomere maintenance.

*The role of E1A in human cell transformation*

E1A is a multifunctional protein that interacts with numerous cellular proteins involved in proliferation control. 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 this interaction, E1A modulates that activity of the E2F family of transcription factors, thus controlling genes required for entry into S phase (Wange et al., 1995; Paulovich et al., 1997; Sherr, 1996). The amino-terminus binds promiscuous transcriptional co-activators, including p300 (Dorsman et al., 1995; Wang et al., 1995; Goodman and Smolik, 2000). The amino-terminus also binds to a protein that has been termed p400 (McMahon et al., 1998 * ref from Emanuelle's old boss). This protein may be related to p300, based upon recognition of both by common antisera; however, the identity of p400 has yet to be reported. The carboxy-terminal region of E1A binds CtBP, a cellular protein, which has been proposed to recruit histone de-acetylases (Goodman and Smolik, 2000).

To map functions of E1A that are essential for it to promote human cell transformation, we used a series of well-characterized deletion mutants. Cells were co-infected with Ha-rasV12 and mutant E1A oncoproteins that are defective in binding to cellular proteins. Engineered cells were tested for the ability to form

colonies in semisolid media and, in the event of positive soft-agar assays, for tumor formation in animals.

A truncated E1A protein consisting of only the amino-terminal 143 amino acids fails to bind CtBP (Boyd et al., 1993; Meloni et al., 1999). However, this mutant is fully capable of cooperating with MDM2 and H-RasV12 both for colony formation in soft-agar (Fig. 4A) and for tumorigenesis in mice (not shown). Expression of E1A-ΔCR2, a mutant incapable of binding Rb (Samuelson and Lowe, 1997), in combination H-RasV12 invariably led to senescent-like growth arrest (irrespective of the presence or absence of MDM2), indicating that interaction between E1A and Rb-family proteins is essential for transformation. Loss of binding to p300 also compromised the ability of E1A to cooperate with either ras alone or ras and MDM2 (Fig. 4A). Finally, an E1A protein that was missing residues 26-35 was also incapable of transformation, indicating that p400 binding is also a critical component of the oncogenic potential of E1A. In all cases, Western blotting with E1A antisera indicated that transformation-defective mutants were abundantly expressed (Fig. 4B). Considered together, these results suggest that E1A functions in human cell transformation through concerted effects on multiple cellular pathways, including Rb, p300 and p400.

**Discussion**

Transformation of normal human cells into cancer cells is a multi-step process, which occurs through combined activation of cellular oncogenes and

inactivation of tumor suppressor pathways (reviewed Paulovich et al., 1997). Most of what we know of this process has come from the study of transformation of rodent cells *in vitro* and from studies of animal models *in vivo*. These have been invaluable to our understanding of neoplastic transformation and of the biology of oncogenes and tumor suppressor. However, it has long been clear that these models do not perfectly recapitulate the process of tumor development in humans. An early indication of this fact was the inability to transform normal human cells in culture with 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). This has opened the doors to the creation of a variety of defined human cancer models, to a detailed study of the cellular pathways that are required for the transformation of normal human cells and ultimately to an understanding of the differential requirements for transformation in humans and in model organisms. This information may provide critical insights as rationally designed anti-cancer therapies move from successful application in animal models to use in humans.

Here, we report that normal human fibroblasts can be transformed into cancer cells by combined expression of adenovirus E1A, activated Ha-rasV12 and MDM2. As with previous successes in human cell transformation, our model

makes use of a combination of viral and cellular oncoproteins that act in a trans-dominant fashion to alter cellular physiology and achieve tumorigenic growth. In common with previous reports, transformation proceeds with a set of oncoproteins that can negate both Rb and p53 tumor suppressor pathways. Through genetic analysis, 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).

One striking difference between our results and those reported previously is that we find no requirement for telomerase activation to achieve either anchorage-independent growth *in vitro* or tumor formation *in vivo*. The majority of human cancer cells are telomerase positive (Kim et al., 1994), and this is a strong indication that the ability to maintain telomeres is an important step in the development of human cancer. Our results are consistent with a model in which telomere maintenance, much like genetic instability (*** no no no *** Duesberg et al., 1999), is a catalyst of, rather than an essential ingredient for tumorigenic growth. The course of tumor formation in a natural setting is likely to be much more circuitous, and thus exhaust much more proliferative capacity, than is required to achieved transformation by acute introduction of a complete complement of oncogenes.

Consistent with their lack of telomerase, or other telomere maintenance strategies, our *in vitro* engineered tumor cells show continuous erosion of telomeric repeats. This leads ultimately to genetic instability that is typified by our observation of numerous chromosome end-to-end fusions in tumor explants. Previously, DePinho and colleagues reported something of a paradox regarding tumor suppressor roles for telomere depletion. Using mice that lack the telomerase catalytic subunit, they reported that the loss of telomeric repeats indeed inhibited transformation (Blasco et al., 1997b). However, if those mice also lacked the p53 pathway, loss of telomeres could actually be pro-oncogenic (* chin et al????). They proposed that cells that are no longer able to mount a programmed response to telomere depletion, via p53, permit complete telomere depletion, which leads in turn to chromosome fusion and rampant genetic instability (Blasco et al., 1997b). While it is formally possible that this type of genomic instability contributes to tumor formation in our model, the fact that tumors remain polyclonal argues against a strong selection for outgrowth of rare cells that acquire additional oncogenic alterations via genetic instability.

Here, we have begun the process of deciphering a minimal set of cellular pathways, which can be altered to achieve the conversion of normal human cells into cancer cells. Using oncoprotein mutants and genetic complementation, we find that inactivation of Rb and p53 tumor suppressor pathways are critical. Furthermore, we find that the ability of E1A to target p300 and p400 are essential to its ability to function as a human oncogene. It will be of interest to determine

whether MDM2 contributes to the transformation of normal 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 an 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 animal models has been the most critical vehicle 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 human cells.

## Materials and Methods

*Cells*

BJ normal human foreskin fibroblasts were maintained at 5% $CO_2$ in Miniumum Essential Medium with Earle's salts (MEM) supplemented with non-essential amino acids (NEAA) and 10% fetal bovine serum (FBS) (Gibco BRL).The amphotropic packaging cell line, LinX-A (Hannon et al., 1999), and 293T cells were maintained in Dulbecco's modified Eagle culture medium (DMEM), supplemented with 0.01% Na Pyruvate and 10% FBS.

*Retroviral Infection*

pBabe-Puro   Ha-rasV12,   pWzl-neo   E1A,   pHygroMaRX   MDM2,

pHygroMaRX p53$^{175H}$ , pHygroMaRX bcl2, pLPC E1A, WZL hygro Ha-rasV12, WZL neo MDM2 and corresponding empty retroviral vectors were used to individually transfect the amphotropic packaging cell line LinX A. Transfection was performed  by the calcium phosphate method. At 72 hr post transfection viral supernatants were collected, filtered, supplemented with 4 µg/ml polybrene and combined in order to obtain the oncogene combinations described in the text. Where single or two oncogenes were delivered, corresponding empty vectors replaced the oncogenes omitted so that infected cells were resistant to hygromycin, puromycin and neomycin. The proper viral mix was then used to infect BJ cells. After infection, cells were selected with a combination hygromycin ( 50 µg/ml), puromycin ( 1 µg/ml) and neomycin (300 µg/ml) for 7 days. Effective infection was confirmed by western blot analysis and by parallel infection with vectors carrying lacZ.

*Anchorage-independent Growth*

Engineered BJ fibroblasts were analyzed for anchorage-independent growth in semi-solid media.  Approximately $3x10^4$ cells were plated in 0.3% low melting point agarose/growth media onto 60-mm dishes over a 0.5% agarose base.  Fresh top agar was added weekly. Colonies were photographed after 2 weeks.

*Subcutanous tumorigenicity assay*

For tumorigenicity assays eight-week-old immunocompromised athymic

nude mice (Hsd:Athymic nude-nu, Harlan) were used. Cells ($5 \times 10^6$) were resuspended in 100 µl of PBS and injected with a 25-gauge needle into the rear flanks of anaesthetized mice. BJ/ER cells were also injected into nude mice that had been γ-irradiated with 400 rad prior injection and into SCID beige mice (C.B-17/lcrHsd-scid-bg, Harlan). Tumor size was monitored every 5 days. Mice were sacrified when tumors reached a diameter of 1-1.2 cm or after 16 weeks. Tumors were collected sterily and minced. Fragments were immediately frozen in liquid nitrogen for DNA and protein extraction and for telomerase assays. Other tumor fragments were fixed in 10% formalin for histological and immunohistochemical examinations. Finally, some fragments were finely minced, washed in PBS and plated in culture medium for isolation of tumor cells.

*Karyotype analysis*

Metaphase chromosomes were prepared from BJ, engineered BJ cells or cells explanted from tumors and quinacrine banding (QFQ staining) was performed according to standard protocols (Barch et al., 1997)

*Clonality and Telomere 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 BamHI , BamHI plus XhoI or BamHI plus 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 or drug resistance markers. Membranes were hybridized overnight at 65°C in 0.2 M NaPO4, 1 mM EDTA, 7% SDS, 1% BSA in the presence of 15% formamide. Membranes were washed twice in 0.1% SDS, 0.2X SSC and once in 0.1X SSC at 60°C, followed by autoradiography. For telomere length evaluation, 3 ug of genomic DNA were digested with Hinfl and Rsal, resolved in a 0.7% agarose gel and hybridized with the telomeric oligomer (CCCTAA)$_3$ as previously described (Wang et al., 1998). Telomerase activity of BJ infected cells before and after implantation was measured using a PCR-based telomeric repeat amplification protocol (TRAP assay), as previously described ( * kim; Wang et al., 1998)

*Western Blot analysis*

Western blotting was performed essentially as described by Harlow and Lane (1988). Cells were washed with cold PBS and lysed in Laemmli loading buffer. Lysates were heated at 95°C for 10 min. Samples were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were incubated with the following mouse monoclonal antobodies: E1A-specific M73 and M35 antibodies; c-Ha-ras (Ab-1) (Oncogene Research Products); MDM2-spcific ***XXXX????????antibody (a kind gift form A. Levine); bcl2 (C2) (Santa Cruz); p53 (DO-1) (Santa Cruz). Immune complexes were visualized by secondary incubation with a sheep or goat anti-mouse HRP-conjuigated secondary antibodies (amersham). Blots were developed by Enhance chemiluminescence (Amersham).

## Acknowledgements

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## Tables

Table 1.

| Genotype | Tumors |
|----------|--------|
| BJ – NHF | 0/38 |
| E1A/ras | 1*/96 |
| E1A/MDM2 | 0/10 |
| ras/MDM2 | Senesced |
| E1A/ras/MDM2 | 40/56 |
| 293/293T | 24/24 |

## Figure Legends

**Figure 1.  Transformation of normal human fibroblasts by E1A and Ha-rasV12.  A.**  Normal human diploid fibroblasts (BJ) were infected with recombinant retroviruses that direct expression of Ha-rasV12, alone or in combination with c-myc or adenovirus E1A (12S).  Cells were scored for proliferation, senescent morphology and senescence-associated β-galactosidase.  **B.**  Cells were infected with recombinant retroviruses as indicated and assayed for colony formation in semi-solid media.  **C.**  Colonies containing greater than 100 cells were counted from triplicate platings of cells with the indicated genotype.  In this experiment, two different Ha-rasV12 constructs were used.  (H) indicates a vector backbone (pWzl-Hygro) in which ras is linked to a hygromycin marker via an IRES sequence.  (P) indicates a vector backbone (pBabe-Puro) in which ras and the selection marker (puromycin resistance) are expressed from separate transcripts.

**Figure 2.  Conversion of BJ fibroblasts into tumor cells by combined expression of E1A, Ha-rasV12 and MDM2.  A.**  Cells of the indicated genotype were assayed for colony formation in semisolid media.  BJ – BJ fibroblasts; E = E1A; R = Ha-rasV12; M = MDM2; 293T – human 293 cells (transformed embryonic kidney cells that express adenovirus E1A) that have been engineered

to express SV40 large T antigen. Where necessary (in all but the ERM cells), cells were infected with retroviruses carrying no oncogene but with the appropriate resistance markers to make those cells triply puromycin, hygromycin and neomycin resistant. **B.** Colonies resulting from triplicate platings of BJ cells of the indicated genotypes in soft agar were quantified. Standard error from the mean is indicated. **C.** Examples of immunocompromised mice (nude, no irradiation) that have been injected with either control, BJ, fibroblasts or with BJ cells that had been engineered to express E1A (E), Ha-rasV12 (R), and MDM2 (M). **D.** Tumor growth rates from two representative mice injected (both flanks) with BJ/ERM fibroblasts are compared to tumor growth rates in a mouse that had been injected (both flanks) with E1A-expressing 293T cells, as indicated.

**Figure 3. Creation of human tumor cells without telomerase activation. A.** TRAP (telomeric repeat amplification protocol) assays were performed on lysates from telomerase-positive 293 cells, BJ/ERM cells and uninfected BJ fibroblasts. **B.** Tumors were recovered from mice injected with BJ/ERM cells. Tumors were assayed for the presence of telomerase activity using the TRAP assay. To test whether tissue extracts contained inhibitors of any step of the assay procedure, we mixed lysate derived from 1,000, telomerase-positive 293 cells with the tumor extract. This produced a positive signal. For comparison, a similar telomerase assay performed using a mass-equivalent portion of lysate from a human breast tumor is shown. **C.** Karyotype analysis of normal BJ fibroblasts and BJ/ERM cells is shown. No cytogenetic abnormalities are detected in the majority of metaphases examined; however, chromosome end-to-end fusions were detected in some metaphases, even prior to injection into mice. **D.** Representative metaphases from BJ/ERM cells explanted into culture following tumor formation are shown. In virtually every metaphase, we note one or more chromosomal abnormalities, including end-to-end fusions and ring chromosomes. This is correlated with a high degree of cell death during the explantation procedure and may reflect this cell population entering a crisis phase due to telomere depletion.

**Figure 4.  Critical functions of E1A in human oncogenesis.  A.**  Scanning deletion mutants (as indicated) of E1A were tested for cooperation with Ha-rasV12 (in the presence or absence of MDM2).  Growth in semi-solid media provided a primary assay (as shown); however, mutants that were positive in this assay were also tested for tumor formation in mice (with MDM2, in all cases). Cellular proteins, which bind to regions covered by the deletions, are indicated below a diagrammatic representation of E1A.  For comparison, soft agar assays with control, BJ fibroblasts and BJ/ERM cells are shown.  **B.**  Western blotting of lysates from cells infected with E1A mutants was performed to insure that defects in colony formation did not result from a lack of E1A mutant expression.
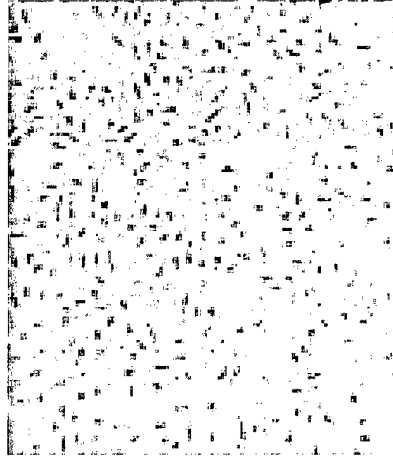
Seger et. al., Fig 1A



Ha-rasV12

c-myc
+
Ha-rasV12

E1A
+
Ha-rasV12

Seger et. al., Fig 1B



BJ p14

BJ/E1A.

BJ/E1A/Ha-rasV12

293

Seger et. al., Fig 1C

Seger et. al., Fig 2A



BJ   EM   RM   ERM   293T

Seger et. al., Fig 2B



Soft Agar Colonies per 30K cells

Genotype

Seger et. al., Fig 2C

con

ERM
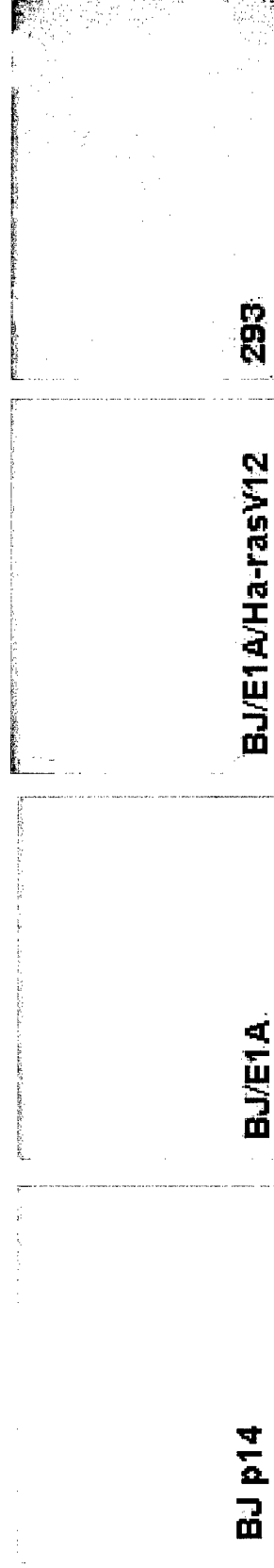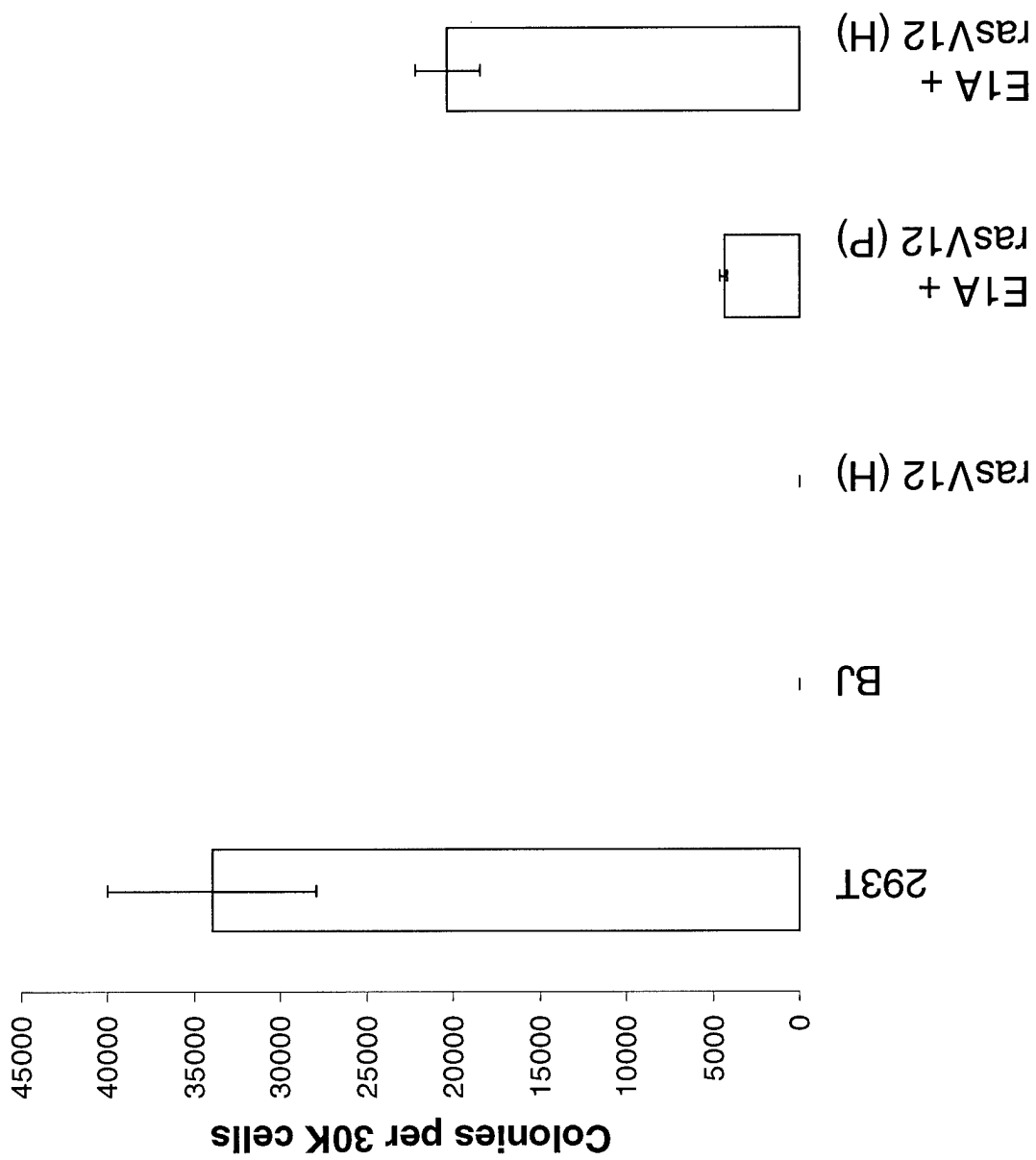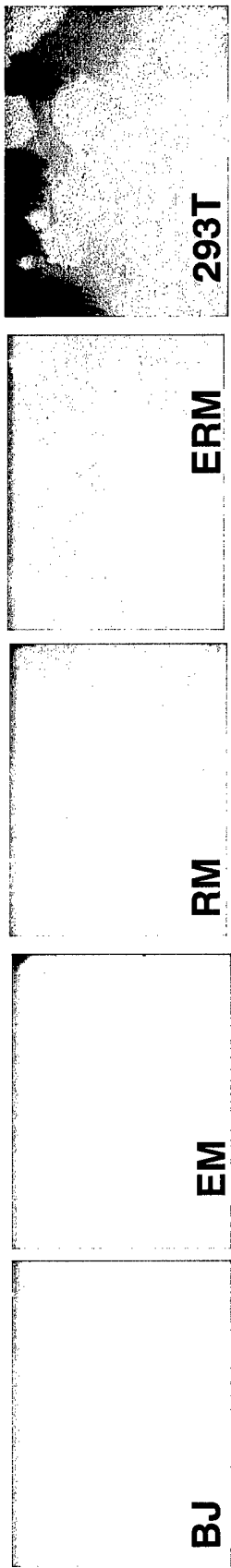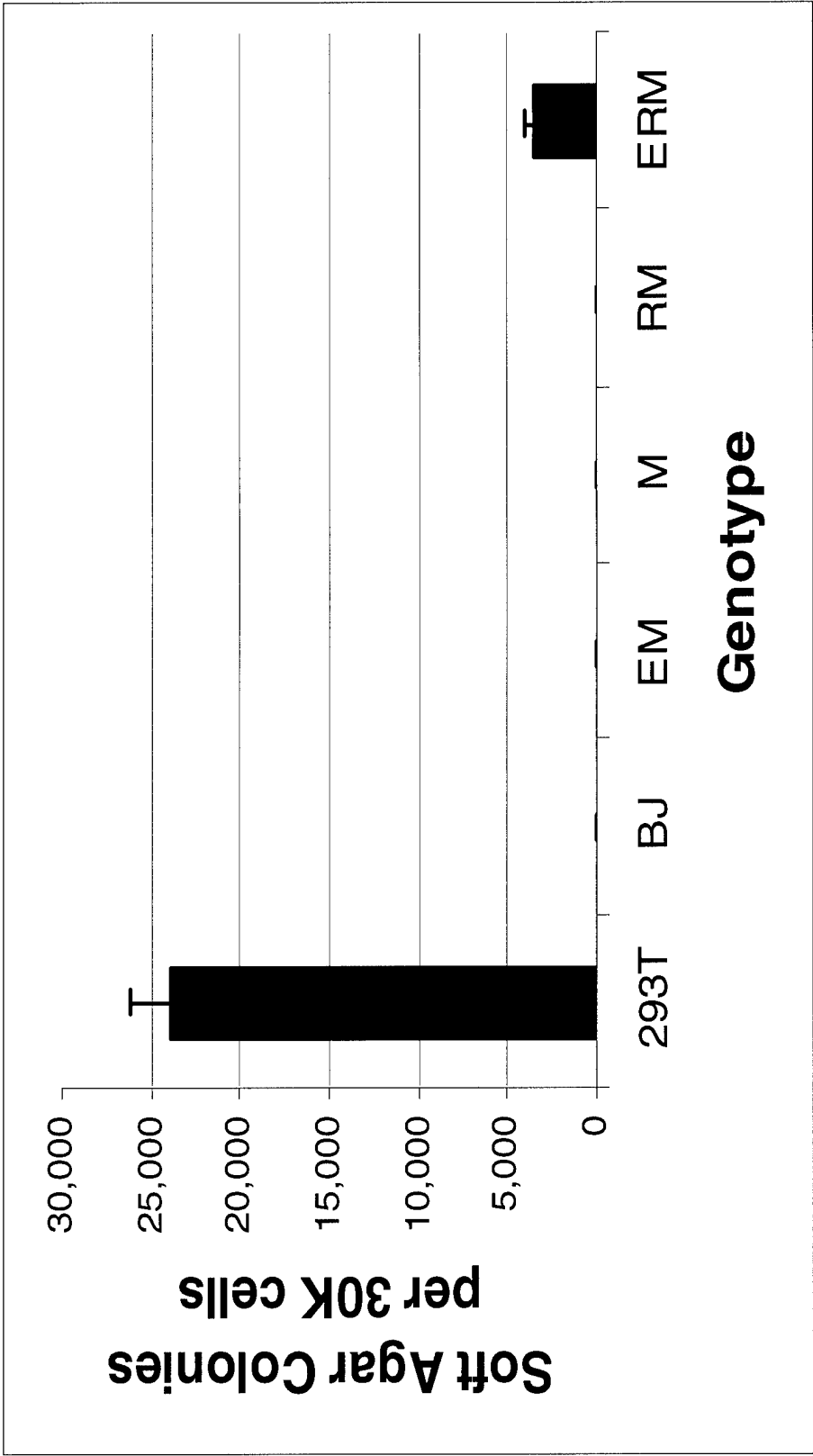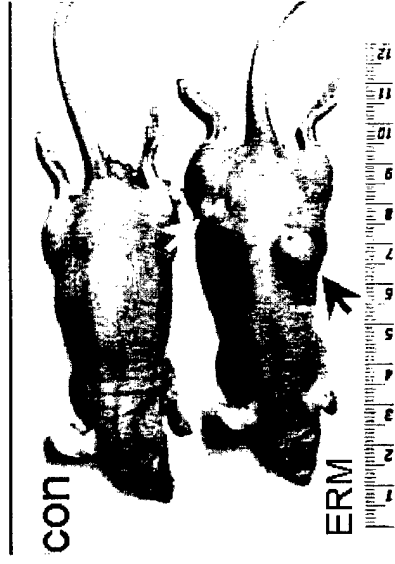
Seger et. al., Fig 2D

Tumor growth rates

293Ta
293Tb
ERM-1496a
ERM-1496b
ERM-1521a
ERM-1521b

tumor diameter (mm)

16
14
12
10
8
6
4
2
0

5    10    15    20    25    30    35    40

time (days post injection)
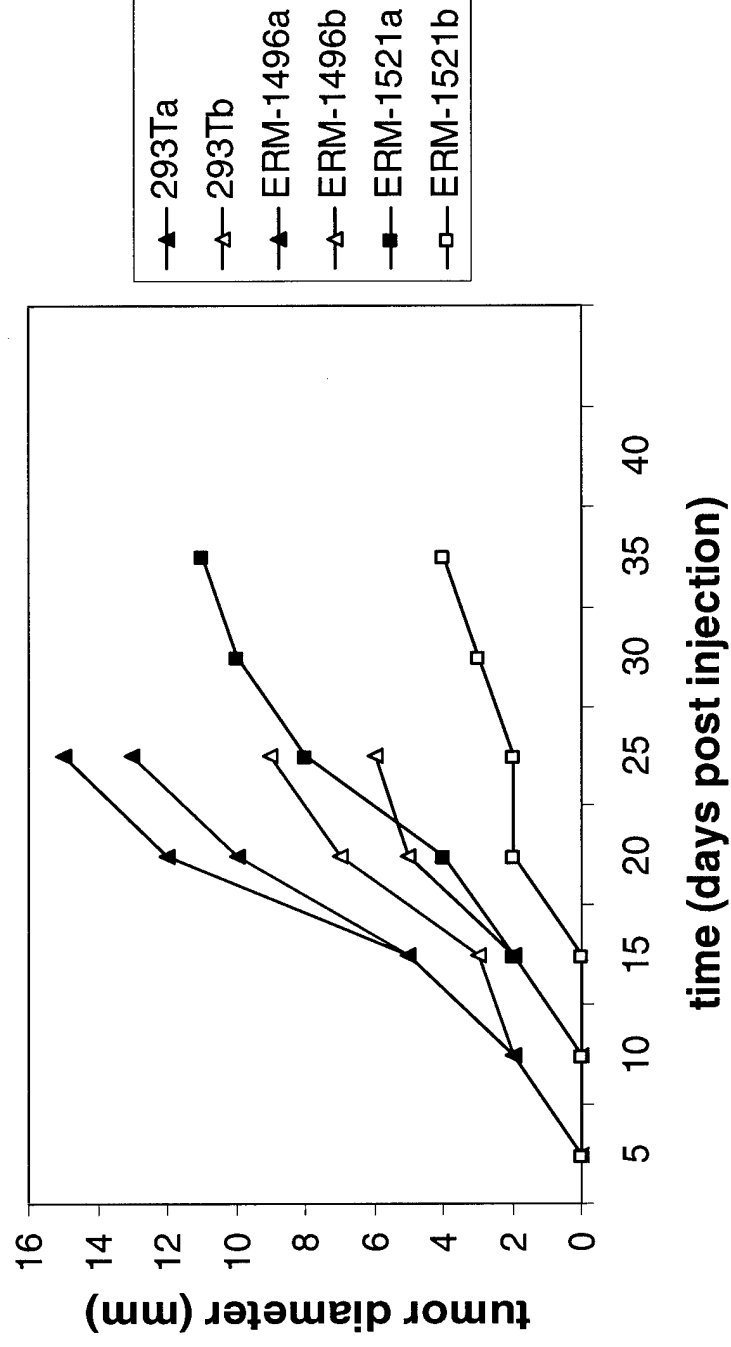
Seger et. al., Fig 3A

BJ 1K
BJ 10K
ERM 1K
ERM 10K
293 1K

Cells pre-injection

Seger et. al., Fig 3B

Human tumor

(ERM tumor

(293 cells + ERM tumor

Tumors

Seger et. al., Fig 3C

BJ E1A/ras/mdm2

BJ

Seger et. al., Fig 4A

Seger et. al., Fig 4B.



Δ48-60/ras
Δ2-24/ras
1-143/ras
Δ64-68/ras
Δ26-35/ras
E1a/ras
BJ
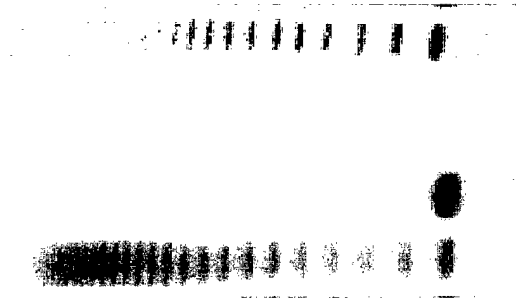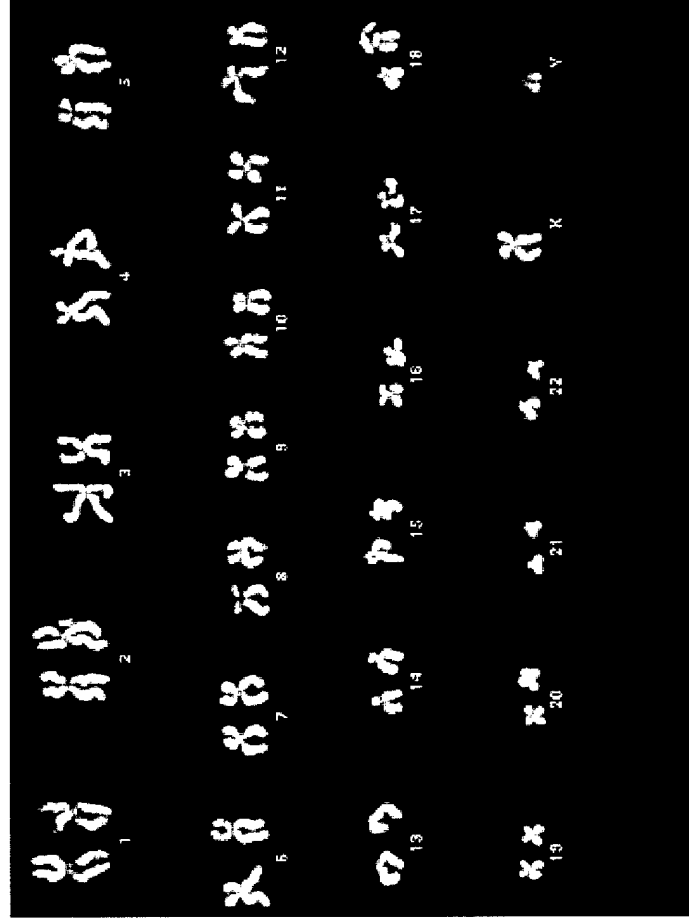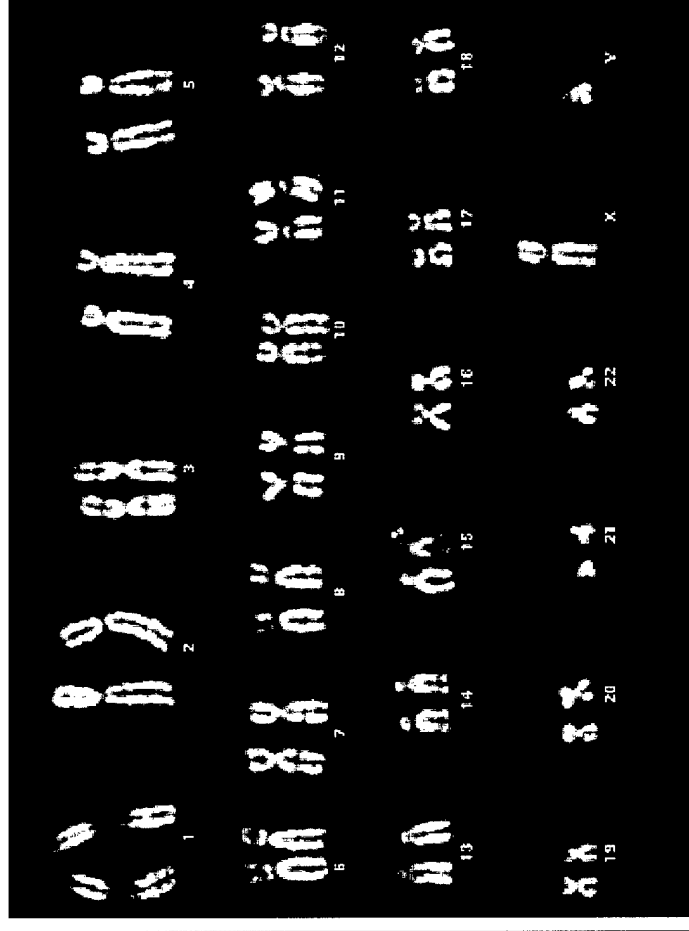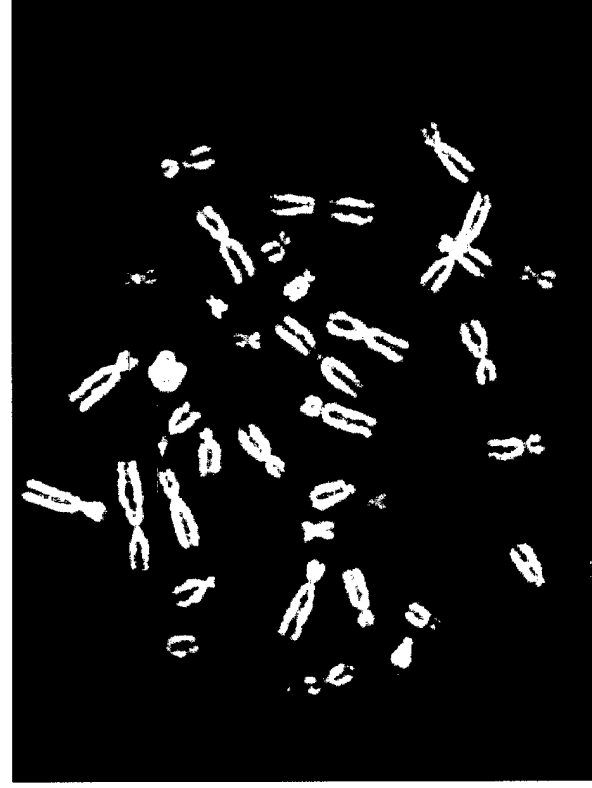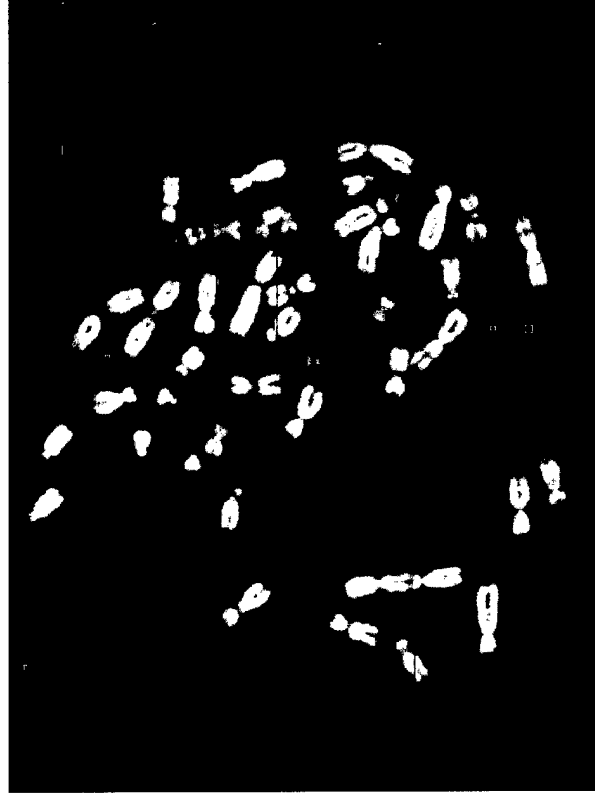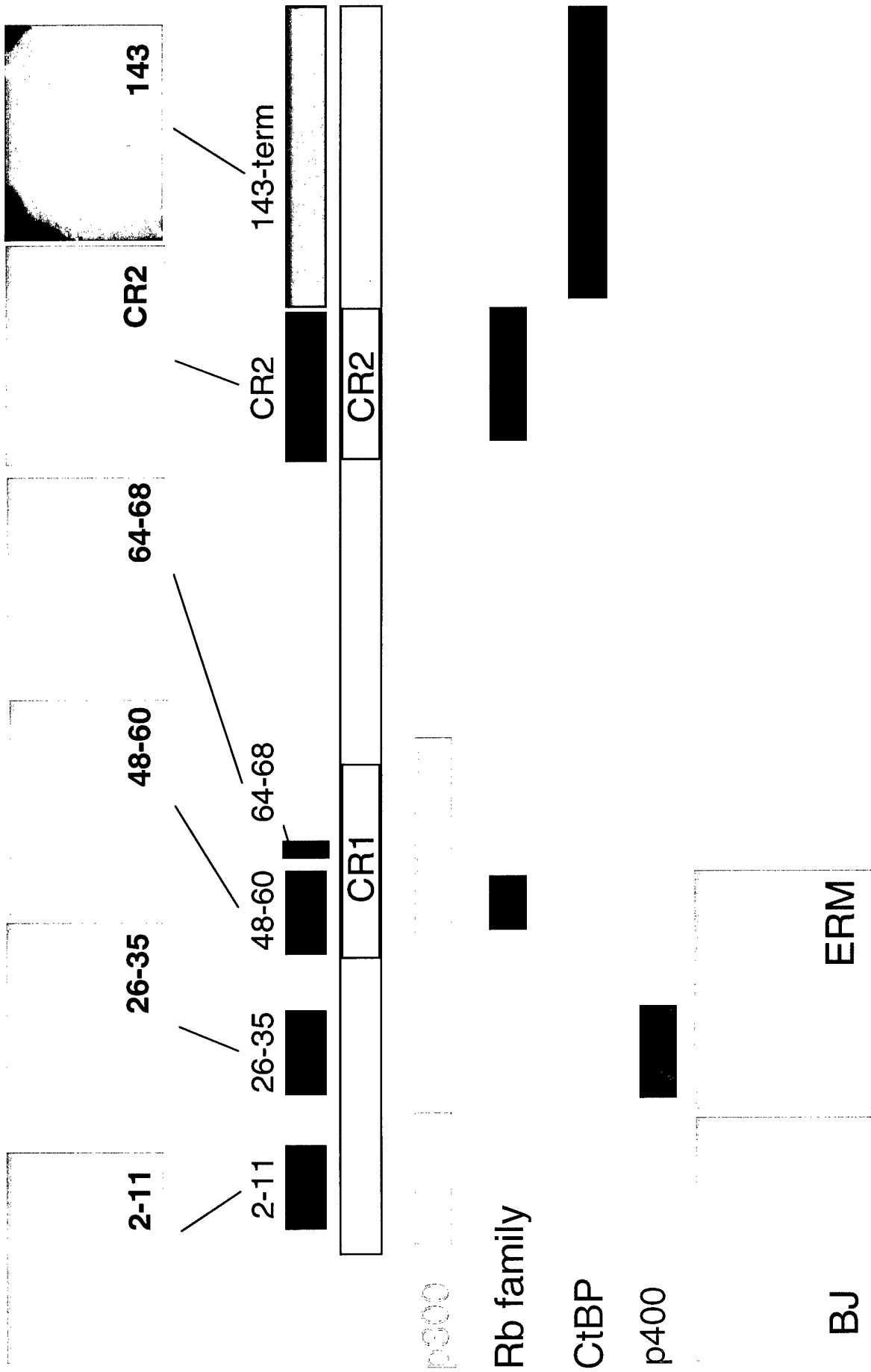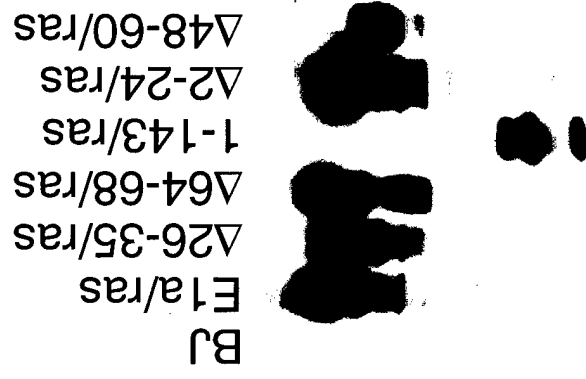
**RNAi in cultured murine cells**

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**Abstract**

In a diverse group of organisms including plants, *C. elegans*, *Drosophila* and trypanosomes, double-stranded RNA (dsRNA) is a potent inducer of sequence-specific gene silencing (reviewed in Hammond et al. 2000). In several model systems, this natural response has been exploited as a powerful tool for the investigation of gene function. Mammalian somatic cells respond to dsRNA through a variety of pathways that ultimately result in a non-specific suppression of gene expression and ultimately cell death (reviewed in Gil and Esteban 2000). Thus, it was somewhat surprising to learn that dsRNA could suppress gene expression in a sequence-specific fashion during early embryogenesis (Wianny and Zernicka-Goetz 2000; Svoboda et al. 2000). Based upon these findings, we tested the possibility that a similar response might be evident in cultured, embryo-derived murine cell lines. In both mouse embyronic stem cells and mouse embyonal carcinoma cells, dsRNA triggers sequence-specific gene silencing without non-specific suppression of gene expression. This response exerts its effect at the post-transcriptional level, suggesting a possible mechanistic relationship with post-transcriptional gene silencing phenomena (e.g. PTGS, RNAi) that have been characterized in other organisms. Harnessing this response as a strategy for negating gene expression in cultured murine cells has the potential to revolutionize the use of somatic cell genetics to understand gene function.

**Background**

The phenomenon of post-transcriptional gene silencing was discovered, somewhat ironically, through an attempt to engineer increased expression of a limiting pigment synthesis gene in petunia (Jorgensen et al. 1996; Que and Jorgensen 1998). However, the evidence that this biological response might be harnessed as a tool came with the discovery that gene silencing could be triggered, at will, by the introduction of dsRNAs into *C. elegans* (Fire et al. 1998).

In C. elegans, RNA interference (RNAi) is associated with a number of remarkable properties (reviewed in Sharp 1999). First, it is a systemic response. Double-stranded RNA can be introduced into the worm by injection, by soaking in a solution containing dsRNA or by feeding worms with E. coli that have been genetically engineered to produce dsRNA. Second, treatment with dsRNA results not only in systemic silencing in the treated animal but also in nearly complete suppression of homologous genes in their F1 progeny.

Many of these properties were reminiscent of transgene-induced silencing and virally induced silencing in plants. It is now clear that, as in C. elegans, PTGS responses in plants can be triggered by double-stranded RNAs. In the case of viruses, these are formed as products of the replication cycle. For multicopy transgenes, the presence of these constructs in complex arrays can

potentially give rise to dsRNA via hairpin formation or via convergent transcription. In the case of cellular genes and dispersed transgenes, the route to dsRNA is less clear; however, genetic evidence suggests that plant RNA-dependent RNA polymerases may catalyze the conversion of primary transcripts to dsRNA.

It has become clear that dsRNA-induced silencing phenomena are present in evolutionarily diverse organisms including plants, fungi and metazoans (reviewed in (Hammond et al. 2001). A combination of genetic and biochemical studies suggest that many of these phenomena share a common mechanism. The prevailing model begins with the conversion of the dsRNA silencing "trigger" into small "guide" RNAs (gRNAs, also known as siRNAs -- Elbashir et al. 2001) that range in size from ~21-25 nucleotides, depending upon the species of origin (Hamilton and Baulcombe 1999; Hammond et al. 2000; Zamore et al. 2000). These RNAs become incorporated into a multicomponent nuclease complex, which uses the sequence of the guide RNAs to identify and destroy homologous mRNAs (Hammond et al. 2000; Zamore et al. 2000). At present neither the signals which trigger systemic silencing nor the mechanisms responsible for their transmission have been elucidated in either plants or animals.

In several systems, dsRNA has been harnessed as a powerful tool for the analysis of gene function. Particularly in C. elegans, RNAi has emerged as the standard protocol for quickly assessing the consequences of loss of gene

function. In fact, programs are underway to create RNAi libraries that can be used to suppress, individually, each of the ~19,000 genes in the worm genome (Fraser et al. 2000; Gonczy et al. 2000). In Drosophila, the first evidence of dsRNA-induced silencing came from the study of embryos (Kennerdell and Carthew 1998), and subsequently, RNAi has proven effective as a gene-silencing tool in cultured cells and in adult insects (Hammond et al. 2000; Clemens et al. 2000; Kennerdell and Carthew 2000).

Despite its utility in diverse systems, the hope of harnessing RNA to study gene function in mammals seemed problematic. Indeed, mammals have evolved robust systems for responding to dsRNAs, specifically as an antiviral defense (reviewed in Williams 1997; Gil and Esteban 2000). In somatic cells, dsRNA activates a variety of responses. Predominant amongst these is PKR, a kinase that is activated by dimerization in the presence of dsRNA (Robertson and Mathews 1996). PKR, in turn, phosphorylates EIF2$\alpha$, causing a non-specific translational shutdown (reviewed in Williams 1997). Double-stranded RNA also activates 2'-5' oligoadenylate polymerase, the product of which is an essential cofactor for a non-specific ribonuclease, RNase L (reviewed in Baglioni and Nilsen 1983).

It has long been clear that the non-specific responses to dsRNA are attenuated during early development. In fact, injection of dsRNA into early-stage mouse embryos (ranging from zygote to the 16-cell stage) can induce sequence-

specific silencing of both exogenous and endogenous genes (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000). Consistent with the possibility that RNAi might extend to mammals, mouse and human homologs of the proteins that participate in this response can be easily identified (reviewed in Hammond et al. 2001).

We sought to determine whether dsRNA could induce sequence specific silencing in cultured murine cells, both to develop this approach as a tool for probing gene function and to allow mechanistic studies of dsRNA-induced silencing to be extended to mammalian systems. We, therefore, attempted to extend previous studies in mouse embryos (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000) by searching for RNAi-like mechanisms in pluripotent, early embryonic cell types.

**Results and Discussion**

We envisioned several possible approaches toward uncovering RNAi responses in mammalian cell lines. First, dsRNAs of greater than 30 nucleotides are required to provoke the PKR response (Vuyisich and Beal 2000); therefore, dsRNAs less than this length might be used as silencing triggers. Second, RNAi might be evident in cells that have been specifically engineered to lack non-specific responses. Indeed, PKR-null cells have been created (Yang et al. 1995; Abraham et al. 1999), and a variety of viral gene products can suppress PKR-

responses in a trans-dominant fashion (see for example, Davies et al. 1993; Cai
et al. 2000; Clarke and Mathews 1995). Third, some embryonic cells naturally
lack non-specific dsRNA responses and might be a context in which specific
dsRNA responses might be revealed. Since dsRNA had been found to
specifically silence gene expression upon injection into early mouse embyos, we
elected to follow the third strategy.

We surveyed a number of cell lines of embryonic origin for the degree to
which non-specific suppression of gene expression occurred upon introduction of
dsRNA. As an assay, we tested the effects of non-specific dsRNA on the
expression of GFP as measured *in situ* by counting fluorescent cells. As
expected, in both human embryonic kidney cells (293) and mouse embryo
fibroblasts GFP expression was virtually eliminated irrespective of the sequence
of the co-transfected dsRNA (not shown). In some pluripotent teratocarcinoma
and teratoma cell lines (e.g., N-Tera2, F9) the PKR response was attenuated but
still evident (not shown); however, in striking contrast, transfection of non-specific
dsRNAs had no effect on the expression of reporter genes either in mouse ES
cells (not shown) or in p19 embryonal carcinoma cells, (Figure 1).

Transfection of P19 embryonal carcinoma cells with GFP in the presence
of cognate dsRNA corresponding to the first ~500 nucleotides of the GFP coding
sequence had a strikingly different effect. GFP expression was eliminated in the
vast majority of co-transfected cells (Figure 1), suggesting that these cultured

murine cells might respond to dsRNA in a manner similar to that which we had

previously demonstrated in cultured, Drosophila S2 cells (Hammond et al. 2000).


To quantify the extent to which dsRNA could induce sequence-specific

gene silencing, we used a dual luciferase reporter assay similar to that which had

first been used to demonstrate RNAi in Drosophila embryo extracts (Tuschl et al.

1999). P19 EC cells were transfected with a mixture of two plasmids that

individually direct the expression of firefly luciferase and *Renilla* luciferase.

These were co-transfected with no dsRNA, with dsRNA that corresponds to the

first ~500 nucleotides of the firefly luciferase or with dsRNA corresponding to the

first ~500 nucleotides of GFP as a control. Co-transfection with GFP dsRNA

gave luciferase activities that were similar to the no-dsRNA control, both in the

firefly/renilla activity ratio and in the absolute values of both activities. In

contrast, in cells that received the firefly luciferase dsRNA, the ratio of firefly to

*Renilla* luciferase activity was reduced by up to 30-fold (250 ng, Figure 2A). For

comparison, we carried out an identical set of experiments in Drosophila S2 cells.

Although qualitatively similar results were obtained, the silencing response was

more potent. At equivalent levels of dsRNA, S2 cells suppressed firefly

luciferase activity to virtually background levels (not shown).


The complementary experiment, in which dsRNA was homologous to

*Renilla* luciferase, was also performed. Again, in this case, suppression of the

expression of the *Renilla* enyzme was approximately 10-fold (Figure 2C). Thus,

the dsRNA response in P19 cells was flexible, and the silencing machinery was able to adapt to dsRNAs directed against any of the reporters that were tested.

We took two approaches to test whether this response was specific for dsRNA. Pre-treatment of the trigger with purified RNAse III, a dsRNA-specific ribonuclease, prior to transfection greatly reduced its ability to provoke silencing (not shown). Furthermore, transfection of cells with single-stranded antisense RNAs directed against either firefly or *Renilla* luciferase, had little or no effect on expression of the reporters (Figure 2B,C). Considered together, these results provided a strong indication that double-stranded RNAs provoke a potent and specific silencing response in P19 embryonal carcinoma cells.

Efficient silencing could be provoked with relatively low concentrations of dsRNA (25 ng/ml of culture media; Figure 2A). The response was concentration-dependent with maximal suppression of ~20-fold being achieved at a dose of 1.5 μg/ml of culture media (Figure 2D)

Silencing was established rapidly and was evident by 9 hours post-transfection (the earliest time point examined). Furthermore, the response persisted without significant changes in the degree of suppression for up to 72 hours (Figures 1,2).

To assess whether the presence of a sequence-specific response to dsRNA was a peculiarity of P19 cells or whether it also extended to normal murine embryonic cells, we performed similar silencing assays in mouse embryonic stem cells. Co-transfection of ES cells with non-cognate dsRNAs (e.g. GFP), again, had no dramatic effect on either the absolute values or the ratios of *Renilla* and firefly luciferase activity (Figure 3). However, transfection with *Renilla* luciferase dsRNA dramatically suppressed *Renilla* luciferase expression.

A key feature of RNAi is that it exerts its effect at the post-transcriptional level by destruction of targeted mRNAs (reviewed in Hammond et al. 2001). To test whether dsRNAs induced silencing in mouse cells via post-transcriptional mechanisms, we used an assay identical to that, which was used initially to characterize RNAi responses in *Drosophila* embyo extracts (Tuschl et al. 1999). We prepared lysates from P19 EC cells that were competent for *in vitro* translation of capped mRNAs corresponding to *Renilla* and firefly luciferase. Addition of non-specific dsRNAs to these extracts had no dramatic effect on either the absolute amount of luciferase expression or on the ratio of firefly to *Renilla* luciferase (Figure 4). In contrast, addition of dsRNA homologous to the firefly luciferase induced a dramatic and dose-dependent suppression of activity. Addition of RNA corresponding to only the antisense strand of the dsRNA had no effect (not shown), comparable to a non-specific dsRNA control, and pre-treatment of the dsRNA silencing trigger with RNAse III reduced its potential to

induce silencing *in vitro*. Considered together, these results suggest that dsRNA can elicit a post-transcriptional gene silencing response in extract from mouse P19 cells.

The discovery that double-stranded RNA could induce gene silencing in organisms as diverse as plants and parasitic protozoans has raised the possibility that RNAi might be a nearly universal mechanism of gene silencing. This notion has been supported the identification of homologs of proteins that participate in the silencing process in virtually all genomes examined to date, with the exception of S. cerevisiae (reviewed in Hammond et al. 2001). The first indications that this response might also extend to mammals came from the observation that injection of dsRNAs into early mouse embryos induced sequence-specific silencing (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000). However, severe limitations on the amount of material available from such experiments has thwarted mechanistic studies.

As an extension of pioneering studies in mouse embryos, we have demonstrated that dsRNA can induce potent and specific gene silencing in mouse embryonic cell lines. Specifically, we have shown that silencing can be induced by long dsRNAs in mouse embryonal carcinoma cell lines and in normal mouse embryonic stem cells. There are several indications that this phenomenon might be related to RNA interference that has been characterized in plants, C. elegans and Drosophia. First, induction of silencing requires double-

stranded RNA, with single-stranded antisense RNA showing negligible effect (Figure 2C). Second, silencing is induced by long (~500 bp) dsRNA triggers, similar to those used in plants, *Drosophila* and *C. elegans*. Third, *in vitro* studies suggest that silencing occurs at the post-transcriptional level (Figure 4). However, final placement of the phenomenon reported here within the pantheon of dsRNA-induced silencing mechanisms will require a characterization of the protein and/or ribonucleoprotein machinery, which enforces suppression.

Our results raise the possibility that, as in several model systems, RNAi might eventually be harnessed as a tool for probing gene function in mammalian cells. To date, suppression of gene expression is more complete in Drosophila cells than in mouse cells (see Figure 2A,B). Furthermore, it is as yet unclear whether RNAi in mammals will suffer from specificity problems similar to those that have been postulated for antisense RNAs. However, the finding that dsRNAs have sequence-specific silencing activity in pluripotent, embryonic murine cells could ultimately ignite a revolution in somatic cell genetics and in the methodologies used for engineering loss-of-function mutations in whole animals.

**Materials and Methods**

*Cell Culture*

P19 mouse embryonic carcinoma cells (ATCC: CRL-1825) were cultured in α-MEM (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum

(FBS) and 1% antibiotic/antimycotic solution (Gibco BRL). Mouse embryo stem

cells were cultured in DMEM containing ESGRO (Chemicon) according to the

manufacturer's instructions.

*RNA Preparation*

For the production of dsRNA, transcription templates were generated by

polymerase chain reaction such that they contained T7 promoter sequences on

each end of the template (see (Hammond et al. 2000). RNA was prepared using

the RiboMax kit (Ambion).  Firefly and Renilla luciferase mRNA transcripts were

synthesized using the Riboprobe kit (Promega) and were gel purified before use.

*Transfection and Gene Silencing Assays*

Cells were transfected with indicated amounts of dsRNA and plasmid DNA using

FuGENE6 (Roche) according to the manufacter's instructions.  Cells were

transfected at 50-70% confluence in 12-well plates containing either 1 or 2mL of

medium per well.  Dual luciferase assays (Promega) were carried out by co-

transfecting cells with plasmids containing firefly luciferase under the control of

SV40 promoter (pGL3-promoter, Promega) and Renilla luciferase under the

control of the SV40 early enhancer/promoter region (pSV40, promega).  These

plasmids were co-transfected using a 1:1 or 10:1 ratio of pGL3 (250ng/well) to

pRL-SV40. Both ratios yielded similar results. For some experiments, cells were transfected with a vector that directs expression of a green fluorescent protein (GFP)-US9 fusion protein (Kalejta et al. 1999). RNAi in S2 cells was performed as previously described (Hammond et al., 2000).

*In vitro translation and in vitro silencing assays*

Logarithmically growing cells were harvested in PBS containing 5 mM EGTA washed twice in PBS and once in hypotonic buffer (10 mM HEPES pH 7.3, 6 mM β-mercaptoethanol). Cells were suspended in 0.7 packed-cell volumes of hypotonic buffer containing *Complete* protease inhibitors (Boehringer) and 0.5 units/ml of RNasin (Promega). Cells were disrupted in a dounce homogenizer with a type B pestle, and lysates were centrifuged at 30,000g for 20 min. Supernatants were used in an *in vitro* translation assay containing capped (m7G(5')pppG) Firefly and Renilla luciferase mRNA. Five microliters of extract was mixed with 100ng of Fifefly and Renilla mRNA along with 1ug dsRNA (or buffer), 10mM DTT, .5mM Spermidine, 200mM Hepes, 3.3mM MgOAc, 800mM KOAc, 1mM ATP, 1mM GTP, 4 units of RNasin (Promega), creatine phosphate (215μg), creatine phosphate kinase (1μg), and 1mM amino acids (Promega). Reactions were carried out for one hour at 30 degrees and quenched by adding 1x PLB. Extracts were then assayed for luciferase activity.

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**Figure Legends**

**Figure 1. RNAi in P19 embryonal carcinoma cells.** 10-cm plates of P19 cells were transfected using 5 ug of GFP plasmid and 40 ug of the indicated dsRNA (or no RNA). Cells were photographed by fluorescent and phase constrast microscopy at 72 hours after transfection; silencing was also clearly evident at 48 hours post-transfection.

**Figure 2. RNAi of firefly and *Renilla* luciferase in P19 cells. A.** P19 cells transfected with plasmids that direct the expression of firefly and *Renilla* luciferases and dsRNAs 500mers (25 or 250ng, as indicated), that were homologous to either firefly luciferase mRNA (dsFF) or non-homologous (dsGFP). Luciferase activity were assayed at various times after transfection, as indicated. Ratios of firefly to renilla activity are normalized to dsGFP controls.
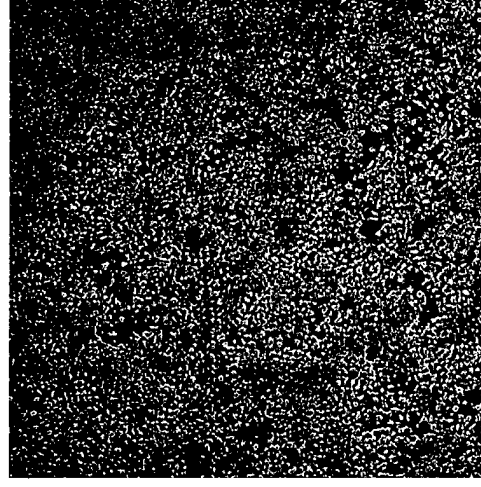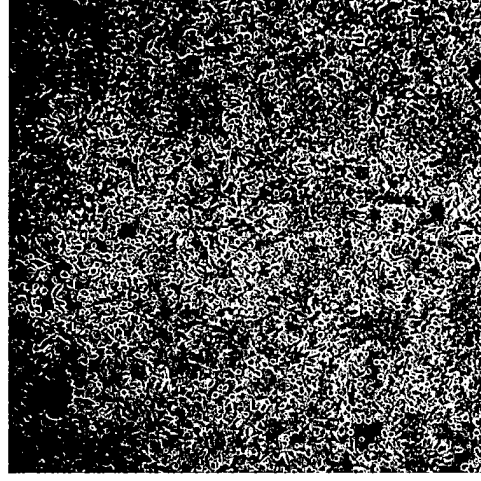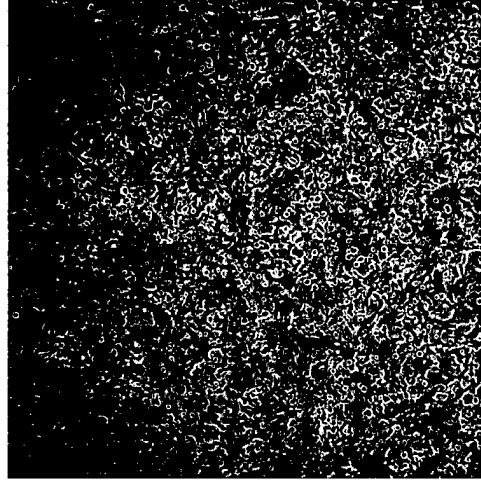
Standard deviations from the mean are shown. **B., C.** P19 cells in 12-well

culture dishes (2 mL media) were transfected with .25 ug of a 9:1 mix of pGL3-

Control and pRL-SV40 as well as 2 ug of the indicated RNA. Extracts were

prepared 9 hours after transfection. **B.** Ratio of firefly to renilla luciferase is

shown. **C.** Ratio of renilla to firefly luciferase is shown. Values are normalized

to dsGFP. The average of three independent experiments is shown; error bars

indicate standard deviation. **D.** The indicated amounts of dsRNA were

transfected into P19 cells (12 well format, 2 mL media per well) along with .25 ug

of a 9:1 mix of pGL3-Control and pRL-SV40. An unrelated plasmid (a CD8

expression vector) was added so that all cells received a total of 1.75 ug of

nucleic acid. The ratio of firefly to renilla luciferase values is indicated, and is

normalized to the no-RNA control. The average of three independent

experiments is shown; error bars indicate standard deviation.

**Figure 3. Specific silencing of renilla luciferase expression by dsRNA in
murine embryonic stem cells.** Mouse ES cells in 6-well culture dishes (2 mL

media) were transfected with the indicated amounts of dsRNA along with .25 ug

each of pGL3-control and pRL-SV40. Extracts were prepared and assayed 24

hours after transfection. Ratios of FF/REN are normalized to dsGFP

transfections.

**Figure 4. dsRNA induces silencing at the post-transcriptional level.** P19

cell extracts were used for *in vitro* translations of *Firefly* and *Renilla* luciferase

mRNA (100ng each). Translation reactions were programmed with various

amounts of dsRNA 500mers, either homologous to firefly luciferase mRNA

(dsLUC) or non-homologous (dsGFP). Luciferase assays were carried out after

a one hour incubation at thirty degrees. Ratios of firefly to renilla activity are

normalized to no dsRNA controls. Standard deviations from the mean are

shown.

Paddison et al., Fig 1



GFP dsRNA

Ren dsRNA

No dsRNA

# Paddison et al., Fig 2A

Paddison et al., Fig 2BC

**B.**



**C.**

# Paddison et al., Fig 2D

# Paddison et al., Fig 3

Paddison et al., Fig 4

Comparison of 21-22nt RNAs and long RNAs in P19 cells, 9 hours