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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

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.

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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 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 has evolved rapidly with the aid of molecular techniques and the use of mouse models to determine oncogenes and tumor suppressors and begin to organize these products into molecular networks to better understand proliferation control in mammalian systems. It is clear, however, that our understanding of the transformation process is more complete in rodent cell systems than human cells; this was made clear by the fact that nearly twenty years passed between publication of the first rodent cell transformation model and the first human cell transformation model.

One potentially critical difference between the requirements for human cell transformation and rodent cell transformation is the expression of the telomerase enzyme, specifically the rate-limiting catalytic subunit, hTERT. In rodent cells, hTERT is ubiquitously expressed in both the somatic and germ cell lines, whereas it is only expressed in the germ cell line in humans (reviewed in McEachern et al., 1994). The Weinberg lab has reported the importance of telomerase in a transformation model that includes co-expression of hTERT, SV40 large and small T antigens, and constitutively activated Harvey ras (V12) (Hahn et al., 1999; Elenbaas et al., 2001; Hahn et al., 2002). Indeed, the majority of human tumors are telomerase positive, thus highlighting the importance of telomerase activation during the course of tumorigenesis (Kim et al., 1994).

In contrast to the Weinberg model, we have shown that primary human fibroblasts could be transformed in the absence of direct introduction of hTERT or an oncogene previously shown to activate telomerase by the co-expression of adenovirus E1A, MDM2, and RasV12. This model initially evolved from the well-characterized ability of E1A to over-ride or bypass the phenomenon of ras-induced senescence in both primary rodent and human fibroblasts (de Stanchina et al., 1998; Serrano et al., 1997), thus allowing high levels of ras expression, a seemingly essential component of all previously established transformation models. In addition, it had been previously shown that primary mouse embryo fibroblasts (MEF) could be transformed by the co-expression of E1A and Ha-RasV12 (Ruley, 1983). More interestingly, however, was our observation that primary human fibroblasts co-expressing E1A and Ha-RasV12 were capable of anchorage-independent growth in soft agar, an *in vitro* test of transformation.

Much to our disappointment, human fibroblasts co-expressing E1A and Ha-RasV12 were unable to result in tumor formation in nude or SCID mice, indicating that additional genetic alterations would be required to convert these *in vitro* transformed fibroblasts into cells capable of tumorigenesis *in vivo*. It had been previously shown that although wild-type MEF could be transformed by co-expression of E1A and Ha-RasV12, p53-/- MEF were more readily and efficiently transformed by this oncogene combination (Lowe et al., 1993). We therefore sought a third genetic hit that would further disrupt and disarm the p53 pathway. The most rational choice was MDM2, the negative regulator of p53. As predicted, human fibroblasts that had been acutely engineered to express E1A, MDM2, and Ha-RasV12 (ERM) simultaneously were capable of both robust colony formation in soft agar and tumor formation in nude mice.

At the time of this proposal (June 2000), we were in the early stages of characterizing this E1A-based transformation model. We now have a rather complete picture as to what is happening in these cells and tumors through key collaborations with

Roberta Maestro (CRO-National Cancer Institute, Aviano, Italy) and Marìa Blasco (National Centre for Biotechnology, Madrid, Spain). We have shown through this model that activation of telomerase is not an obligate event of transformation, however it may play a more downstream role in tumor maintenance. In addition, we have successfully transformed additional primary human fibroblast cell types with this oncogene combination, indicating that this result is not an anomaly of BJ fibroblasts.

Below is an outline of the major results accumulated for each of the tasks within the research proposal. More detailed descriptions and corresponding figures for these results can be found within the manuscript included in the Appendix section.

TASK #1: MODELS FOR HUMAN CELL TRANSFORMATION

As previously stated, we have successfully converted normal human fibroblasts (BJ) into tumorigenic cells through the combined expression of E1A, Ha-RasV12, and MDM2 (ERM). Oncogene expression is mediated through the use of replication-deficient retroviruses that encode each oncogene. Each retrovirus bears a different drug resistance marker (E1A = puromycin, Ha-RasV12 = hygromycin, and MDM2 = neomycin) that allows for the selection of cells expressing specific viruses. In order to engineer these cells in an acute manner, we have adopted a protocol in which all three retroviruses are introduced at once in order to eliminate the probability of secondary mutations that might occur through continued passage of cells in culture during a serial infection protocol.

One interesting aspect of our ERM transformation model is that there is no direct introduction of telomerase, nor the introduction of an oncogene previously shown to activate telomerase expression. We have therefore spent a significant amount of time characterizing this ERM transformation model through analysis of the parental ERM cells, resultant ERM tumors, and cells explanted from ERM tumors. These results are included as a manuscript entitled "Transformation of normal human cells in the absence of telomerase activation" within the appendices of this report. Results of major significance are highlighted below.

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

Cell immortalization has been posited as a landmark occurrence in the series of events in which a normal cell becomes 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 expression of the 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 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). Based upon serial dilutions, 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). Based upon mixing experiments, 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 that are maintained in culture for an extended period of time become telomerase positive. This phenomenon is correlated with increased apoptosis, thereby signaling the possibility of telomere crisis in the absence of an enforced telomere maintenance program.

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

Once we had verified that the ERM-engineered fibroblasts that we had injected were telomerase-negative, we were curious to ascertain the telomerase status of the resultant tumors and determine whether telomerase activation had occurred as a function of *in vivo* tumorigenesis. Telomerase activity was measured utilizing the standard TRAP assay described above on tissue sections obtained from the ERM tumors in immunocompromised mice. Whereas a tissue sample from a human tumor indicated robust telomerase activity in a TRAP assay, the lysate from ERM tumor tissue was telomerase-negative. 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 293 cells. When the 293 cell and ERM tumor lysates were mixed in a TRAP reaction, the result was telomerase positive, indicating that there was no inhibitory component within the tumor lysate, and thus the tumors were below detectable counts for telomerase activity.

Immortal cells are able to maintain telomere length through at least two independent mechanisms, with the most common being telomerase activation (Kim et al., 1994; Bodnar et al., 1998). However, alternative, recombination-based pathways of telomere maintenance (ALT) have also been described (Dunham et al., 2000; Bryan et al., 1997a; Hoare et al., 2001; Bryan et al., 1997b). Thus it was possible that BJ-derived tumor cells had invoked a mechanism of telomere maintenance that could not be detected through telomerase activity. We therefore analyzed telomere length by telomeric restriction fragment (TRF) analysis (Harley et al., 1990) in BJ cells prior to infection, BJ cells engineered to express E1A/Ha-RasV12/MDM2 passaged *in vitro,* and in tumors that formed upon injection of these engineered cells. In all cases, continuous telomere erosion was evident and correlated with the proliferation of these cells *in vitro* or *in vivo.*

C. The karyotypes of explanted BJ/ERM cells indicate chromosomal abnormalities consistent with continuous telomere erosion

As noted above, BJ cells are engineered to express E1A, Ha-RasV12, and MDM2 through simultaneous co-infection. 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. Examination of cells that are explanted into culture following tumor formation, however, reveals numerous genetic abnormalities. 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. These types of genetic abnormalities are a characteristic outcome of telomere depletion and similar to those seen in the karyotypes of hTERT-/- 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.

D. Multiple primary human fibroblast lines can be transformed by coexpression of E1A/MDM2/Ha-RasV12

While BJ cells are considered to be normal primary human fibroblasts, several groups have shown that these cells have unusually long telomeres (Bodnar et al., 1998) and thereby are capable of an increased number of potential population doublings as compared to most primary human fibroblast types. Therefore, in order to verify that E1A/MDM2/Ha-RasV12-mediated transformation is not unique to BJ fibroblasts, we performed the same experiments in several additional human fibroblast cell lines, including HSF43, WI-38, and DET551. As had been seen in BJ fibroblasts, when these additional fibroblasts lines were engineered to co-express E1A/MDM2/Ha-RasV12, they were capable of anchorage-independent growth in soft agar. Similarly, these triple-infected fibroblasts were capable of tumor formation when injected into immunocompromised mice. We can thereby conclude that this E1A/MDM2/Ha-RasV12-mediated transformation model is not unique to BJ fibroblasts, and can be used to transform several types of primary human fibroblasts with relatively high efficiency.

TASK #2: GENETIC REQUIREMENTS FOR THE HUMAN CELL TRANSFORMATION

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

In the initial proposal, we had planned to determine the essential pathways in an E1A-based transformation model by complementing E1A functional mutants with retroviruses driving the expression of full length or fragments of viral or cellular oncogenes. We had previously shown that substitution of wild-type E1A with various functional mutants, such as those defective for the ability to bind pRB or p300, were unable to cooperate with MDM2 and Ha-RasV12 to result in anchorage-independent growth in soft agar. Western blot analysis verified that this effect was not due to a lack of E1A expression since all of the mutants were expressed at levels similar to that seen in BJ/ERM cells. By trans-complementation of these mutants with a selection of viral or cellular oncoproteins, I had hoped to further determine the pathway requirements for E1A-mediated transformation. Although this technique of trans-complementation has been used to determine many functional interactions of proteins, recent developments in our laboratory during the past few months have resulted in the development of a very powerful tool that would be a much more effective method of determining the precise pathway interactions of E1A in human cell transformation.

A majority of our lab studies the phenomenon of RNA interference (RNAi), with one group working to elucidate the mechanism of this gene silencing and another group working towards the development of molecular techniques that allow us to harness this powerful tool and use it for targeted gene silencing in mammalian (specifically human) cell systems. Recently, our lab has shown that short hairpin RNAs (shRNAs) can be

utilized to induce sequence-specific gene silencing in mammalian cells (Paddison et al., 2002), and even more importantly for the purposes of this project, in primary human fibroblasts such as IMR90. By utilizing shRNA retroviruses, we can determine the pathway interactions required by E1A in human cell transformation by creating virtual knockouts of E1A binding partners and targets.

For example, shRNA targeting of the cellular protein CtBP (a negative regulator of transformation) in cells expressing ERM should not affect the ability of these cells to proliferate or form colonies in soft agar, similar to the result we observe when using the CtBP binding-deficient C-terminal truncated version of E1A. We would also predict that shRNA targeting of pRB would promote ERM-mediated transformation since the ability of E1A to bind and sequester pRB, thus inactivating it, has been shown to be vital to its role in transformation. This technique, once validated with these controls, can then be used to examine the potential importance of additional E1A targets, namely p300, CBP, p400, and TRRAP. One caveat to this type of experiment is that suppression of an essential gene (required for cell survival) would yield an uninformative result. For example, we predict that shRNA-induced silencing of CtBP will not affect ERM-mediated transformation based upon positive transformation data I have obtained using the E1A-143 mutant. However, if CtBP turns out to be an essential gene, shRNA targeting of CtBP would kill the cells and yield an uninformative result. For these analyses, the growth rates of each engineered cell line will be monitored when placed on a 3T3 maintenance schedule compared to the original BJ/ERM cells. I will also test each engineered cell line for the ability to sustain anchorage-independent growth in soft agar, and if the cells are viable, for tumorigenesis in vivo.

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

Analysis of the function of MDM2 in mediating E1A + Ras tumorigenesis

At the time of my proposal, I had not yet evaluated the expression of the E1A functional mutants in the context of MDM2 over-expression. Since MDM2, like E1A, interacts with the transcriptional coactivators p300 and CBP, I was curious to determine if co-expression of MDM2 with the N-terminal deletion mutants of E1A (defective for p300/CBP binding) would partially rescue the inability of these mutants to cooperate with Ha-RasV12 to result in anchorage independent growth. Much to my surprise, MDM2 was unable to rescue any of the E1A functional mutants to result in colony formation in soft agar. These results indicate that the interactions of MDM2 with p300/CBP are unable to compensate for the loss of E1A to interact with these same coactivators.

Analysis of the ability of anti-apoptotic genes to cooperate with E1A and E1A functional mutants in tumorigenesis

While E1A is known to act as a proliferative signal, overexpression is highly cytotoxic and results in the induction of programmed cell death. Adenoviruses evade this internal apoptotic mechanism through expression of the anti-apoptotic genes expressed by the E1B region. Cells transformed by the E1A, however, do not have this adenovirus-encoded protection from cell death. Since E1A-induced apoptosis is mediated through induction of the p53 pathway, it is reasonable to hypothesize that MDM2, as the negative regulator of p53, would be capable of abrogating an E1A-stimulated apoptotic response in order to promote tumorigenesis.

In order to pare down the role of MDM2 in the ERM transformation model, MDM2 was substituted with a dominant-negative form of p53 (175H) or Bcl-2. Substitution of MDM2 with dn-p53(175H), which is capable of both bypassing p53-mediated growth arrest and acting as an anti-apoptotic, would indicate if the ability of MDM2 to interact with p300/CBP is vital to its transforming ability or if there are some additional, unknown interactions. Furthermore, complementation of MDM2 with the anti-apoptotic gene Bcl-2 would indicate whether MDM2 is functioning solely in an anti-apoptotic function in mediating transformation.

We have obtained preliminary data for these experiments. Substitution of MDM2 with either dn-p53(175H) or Bcl-2 allows for anchorage-independent growth in soft agar, although it is interesting to note that the colonies formed by Bcl-2 expressing cells arise at a much slower rate and are smaller in size than those formed by either ERM or ER + dn-p53 (175H). Initial in vivo tumorigenesis assays in nude mice have indicated that E1A/dn-p53(175H)/RasV12 cells are capable of tumor formation whereas cells expressing Bcl-2 do not result in tumors. We are repeating these injections in order to obtain a larger sample size. In addition, tumor growth rates of the dn-p53(175H) tumors versus ERM tumors will be monitored in order to determine any differences.

Analysis of the ability of MDM2 binding mutants to cooperate with E1A in tumorigenesis

The scope and necessity of this section for further determination of the role of MDM2 in E1A is dependent on further clarification of the data obtained in the above section. The original purpose of proposing to test MDM2 binding mutants for the ability to cooperate was to determine the minimum interaction(s) required for MDM2 to be functional for transformation in this model. If, however, we find that dn-p53(175H) is sufficient for promoting transformation while Bcl-2 is not, we will have already narrowed the interactions provided by MDM2 to the p53 pathway. In addition, if ERM tumors have increased growth rates when compared to those of dn-p53 (175H) tumors, we can infer that additional interactions of MDM2 beyond the p53 pathway contribute to the rate of tumor formation.

Use of shRNAs to determine functional interactions and pathways necessary for the role of MDM2 in the ERM transformation model

In light of the preliminary data that indicate that the interaction of MDM2 with the p53 pathway provides a vital component to the E1A/MDM2/Ha-RasV12 transformation model, I propose a set of experiments utilizing shRNAs to specifically target expression of p53. One would propose that the selective silencing of p53 through this type of experiment would actually allow for more efficient transformation by E1A and Ras, as had been seen in MEFs null for p53 expression. Based upon the initial results in which MDM2 was substituted with either dn-p53 (175H) or Bcl-2, we can conclude that p53 is not functioning only through an apoptotic pathway. However, I will need to verify that the cells expressing Bcl-2 are in fact more resistant to apoptosis. This can be tested by viability assays in which one monitors cell survival during a time course exposure to apoptotic stimuli such as serum starvation, DNA damaging agents, and chemotherapeutic drugs such as adriamycin.

KEY RESEARCH ACCOMPLISHMENTS

TASK #1: Creation of Human Tumor Cell Models

- Co-expression of E1A and Ha-RasV12 permits anchorage-independent growth.
- Cells co-expressing E1A and Ha-RasV12 are insufficient to promote tumorigenesis.
- Combined expression of E1A, Ha-RasV12, and MDM2 transforms normal human cells into tumor cells.
- Human fibroblasts transformed by co-expression of E1A, Ha-RasV12, and MDM2 lack telomerase activity.
- Tumors derived from fibroblasts expressing E1A, Ha-RasV12, and MDM2 lack telomerase activity.
- Karyotypes of cells explanted from E1A/Ha-RasV12/MDM2 tumors reveal chromosomal abnormalities characteristic of telomere depletion.
- This E1A/Ha-RasV12/MDM2 transformation model can be used to transform multiple types of primary human fibroblasts.

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.

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. (under review, 2002). Transformation of normal human cells in the absence of telomerase activation.

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

CONCLUSIONS

In conclusion, in the time since this proposal was first submitted, we have made significant strides in understanding the genetic requirements for the transformation of human cells. The ERM transformation model is highly reproducible in a variety of human fibroblast types, and we are currently working on applying this model to cells of epithelial origin in order to more effectively recapitulate human breast cancers. This ERM model also provides us with a useful tool through which we can analyze the precise molecular pathways involved in transformation processes through analysis of E1A functional mutants.

In addition to the mutant analysis and complementation experiments that originally existed within this proposal, this system also provides a unique system in which new RNAi techniques can be tested, namely the use of short hairpin RNAs (shRNAs). Use of such technology will assist us in our ultimate goal of determining the minimum pathway requirements in ERM-mediated tumorigenesis.

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Transformation of normal human cells in the absence of telomerase activation

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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, activation of telomere maintenance strategies is not an obligate characteristic of tumorigenic human cells.

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 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.

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 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). 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 $p19^{ARF}$ 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 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 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 (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 co-expression 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

Co-expression 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 (Fig. 1A) (Serrano et al., 1997; de Stanchina et al., 1998). In fact, co-expression 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 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 BJ fibroblasts (normal human foreskin fibroblasts) 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 (Fig. 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 Fig. 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 co-activators, 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 de-acetylases (Goodman and Smolik, 2000).

In order to map the regions and interactions of E1A that are essential for its ability cooperate with Ha-RasV12 in conferring anchorage independent growth to human primary fibroblasts, we used a series of well-characterized deletion mutants for *in vitro* transformation assays (Samuelson and Lowe, 1997). Cells were co-infected 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 Ha-RasV12 for colony formation in soft agar with high efficiency (Fig. 1D). 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 suggested that each was expressed similarly to wild-type, with the exception of the CR2 mutant for which the prevalence of cell death in ras co-infected cells made analysis impossible (Figure 1E). 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 passage BJ fibroblasts co-expressing 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, non-irradiated mouse (Figure 1F). 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 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 et al., 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 pre-injection 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 were simultaneously co-infected 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 co-selected with puromycin, hygromycin and neomycin for ten days and then either plated into soft agar or injected into immunocompromised mice. 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 (see Figs. 1 and 2). Moreover, the triple-infected cells were able to generate tumors when injected subcutaneously into immunocompromised in mice (Fig. 3). 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 (Fig. 3). Tumor formation was observed also when E1A was substituted by the C-terminal deletion mutant E1A-143 (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 (Fig. 3C)

Cell populations remained polyclonal throughout drug selection *in vitro* and tumorigenesis *in vivo* as revealed by Southern blotting analysis (data not shown). 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.

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 expression 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)(Fig. 4A). 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 (Fig. 4A, 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 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 4,5).

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, the lysate from ERM tumor tissue was telomerase-negative (Fig 4B). 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 (Fig 4B).

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 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 4C). To increase the sensitivity of our assay we performed southern blot of the PCR reactions. After southern analysis, one out of 6 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. 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 late during tumor progression.

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 post-crisis late passage BJ/ERM, surviving tumor cells have become telomerase positive (Figure 5A). 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*. Telomeres in early passage BJ cells averaged ~7 Kb in length. These became depleted as BJ cells were engineered 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 the 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 5C,D). 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, when cells explanted from ERM tumors, which had become telomerase-positive *in vitro*, were injected into a second nude mouse, the resultant tumors formed at rates similar to those of primary ERM tumors (data not shown), indicating that telomerase status did not affect the tumorigenicity of ERM cells.

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

As noted above, BJ cells are engineered to express E1A, Ha-RasV12, and MDM2 through simultaneous co-infection. 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 (Figure 6A). Examination of cells that are explanted into culture following tumor formation, however, reveals numerous chromosomal abnormalities (Figures 6B and 6C). 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-depleted telomeres. In some metaphases, we also find ring chromosomes (Figure 6B). 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 6C). 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 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, DET551, SF68 as well as in the human primary mesodermal cells HMSC. Upon co-expression 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 (Figure 7A and B)

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 easily 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 anti-cancer therapies move from successful applications in animal models to use in humans.

Here we report that normal 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 trans-dominant 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 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 can antagonize tumorigenesis. Indeed $Terc^{-/-}$ mice are more resistant to chemical carcinogenesis and to spontaneous tumor development both in a 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 short telomeres actually act as a pro-oncogenic factor under certain conditions (Chin et al., 1999).

Our data suggest that, while changes in telomere biology are undoubtedly important for the course of natural tumor development, telomere maintenance is not required for the creation of human cancer cells by acute alterations in 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.

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 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

BJ normal human foreskin fibroblasts were maintained in Minimum 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), 293T, Detroit 551, WI-38, HSF43, SF68 cells were maintained in Dulbecco's Modified Eagle culture medium (DMEM), supplemented with 0.01% Sodium Pyruvate and 10% FBS. 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.

Retroviral Infection

pBABE-Puro Ha-rasV12, Wzl-Neo E1A 12s, pHygroMaRX mdm2, and corresponding empty retroviral vectors were used to singularly transfect the amphotropic packaging cell line LinX-A. Transfection was performed by the calcium phosphate method. At 72 hours 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. 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 early passage human primary fibroblasts (BJ, Detroit 551, WI-38, HSF43, SF68) and human primary mesodermal cells (HMSC). 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.

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 antibodies: E1A-specific M73 and M58 antibody; 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, dnp53, bcl2), alone or in combination, were analyzed for anchorage-independent growth in semi-solid media. Approximately 10^5 cells were plated in 0.3% low melting point agarose/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 etopically-expressed oncogenes was detemined 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 (Barch et al., 1997)

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 2 chromosomes fused by their p-arms (Robertsonian-like fusions) or 2 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 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. 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, 0.2X SSC and once in 0.1X 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 over-saturation 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)

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

Telomerase assays

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

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'-TTCCTGCACTGGCTGATGAGTGT-3' and hTERT1a, 5'-AGCGTCGGCCCTCTTTTCTCTG-3' were used to amplify a 330 bp fragment spanning exons 3 and 4 of the human TERT sequence. Primers hTERT2s, 5'-ACAGCACTTGAAGAGGGTG-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 cycles with an elongation at 72°C for 30 sec 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.

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Figure Legends

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 co-infected 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 semi-solid 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.

D. A series of well-characterized functional mutants of E1A were co-expressed in BJ fibroblasts with Ha-RasV12 and assayed for the ability to form colonies in semi-solid 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.

E. Western blot analysis indicated that the E1A functional mutants were well-expressed as compared to wild-type, and thus the defects in colony formation were not due to E1A not being adequately expressed.

F. BJ fibroblasts co-expressing E1A and Ha-RasV12 were unable to result in tumor formation in nude mice. There was one instance in which a single tumor formed in the flank of a bilaterally injected mouse, which is denoted as 1*. This tumor arose after a much greater latency period, indicating the possibility for the selection of a mutation *in vivo.*

Figure 2.

MDM2 cooperates with E1A and Ha-RasV12 to promote 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 semi-solid 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.

Figure 3.

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 injected in both flanks 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.

D. A tabulation of tumor formation by BJ/ERM cells in nude mice.

Figure 4.

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 emerge 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 pre-treated 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. For comparison, a similar telomerase assay was 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 tumor samples. Expression was tested by using two primer primer pairs that would allow for 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.

Figure 5.

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 analysed 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.

C. Telomere length distribution of BJ, BJ/ERM and BJ/ERM tumor cells at different passages as determined by quantitative FISH (Q-FISH) using a telomeric 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.

D. 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 EMR cells and in the tumor cell line. Blue: DAPI; Yellow dots: telomeres.

Figure 6.

Karyotypes of ERM cells post-explantation indicate chromosomal abnormalities characteristic of telomere depletion

A. Karyotypic analysis of normal BJ fibroblasts and BJ/ERM cells by G-banding is shown. No cytogenetic abnormalities were detected in the majority of metaphases.

B. 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 during telomere depletion.

C. Quantification and representative image of cytogenetic alterations detected in metaphases from different passage BJ, BJ/ERM and BJ/ERM tumor cells after hybridization with DAPI and a fluorescent Cy-3-labeled telomeric peptide nucleic acid (PNA) probe. Blue, chromosome DNA stained with DAPI; yellow and white dots, TTAGGG repeats. Karyotype analysis is BJ, BJ/ERM and BJ/ERM tumor

cells at different passages is also shown. For each culture, 15 metaphases were evaluated. Notice that the tumor-derived cell lines were severely aneupliod compared with BJ cells at two different passages. ERM p20 and ERM pc cells showed a modest aneuploidy. FRAGM: chromosome fragment; DICENT: dicentric; TRICENT: tricentric; TRANSL: translocation.

Figure 7.

Multiple human fibroblast cell lines can be transformed by the combined expression of E1A, Ha-RasV12, and MDM2

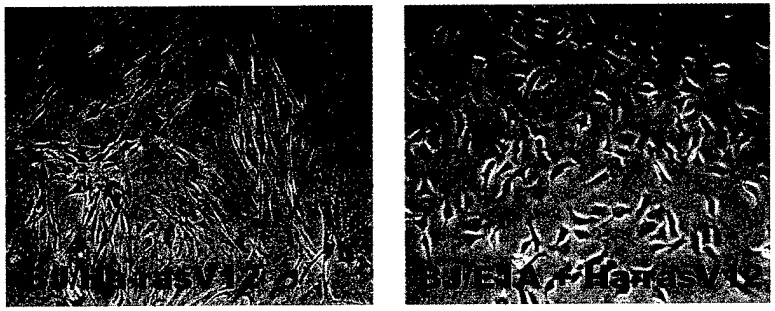
A. Examples of immunocompromised mice (nude, no γ-irradiation) injected with control fibroblasts expressing E1A + Ha-RasV12 or fibroblasts engineered to express the oncogene combination of E1A + Ha-RasV12 + MDM2. As had been seen with BJ fibroblasts, additional human skin fibroblasts (HSF43, WI-38, and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

B. Table quantifying the frequency of tumor formation by different human fibroblast lines expressing ERM.

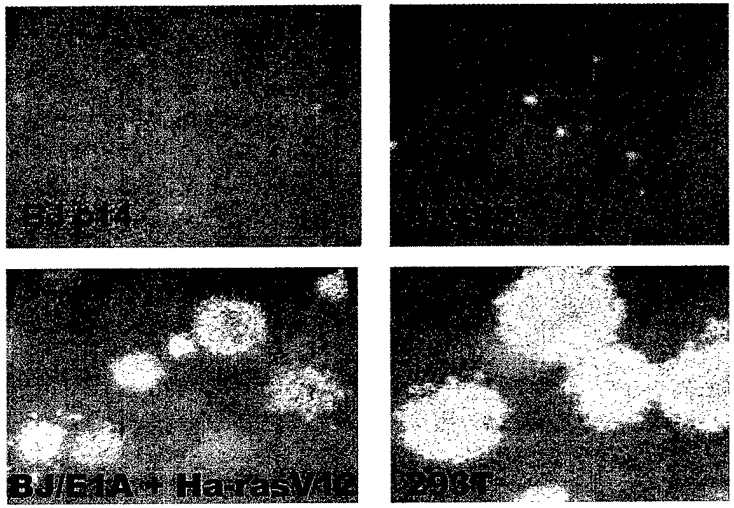
C. RT-PCR was used to detect hTERT expression in tumor samples. Expression was tested by using two primer primer pairs that would allow for the specific amplification of hTERT and not mouse TERT that might be present from contaminating murine cells within the tissue sample. β-actin served as an internal control. Sensitivity of the assay was increased by performing a Southern blot of the PCR reactions for hTERT.

Seger et al., Figure 1A, 1B, and 1C

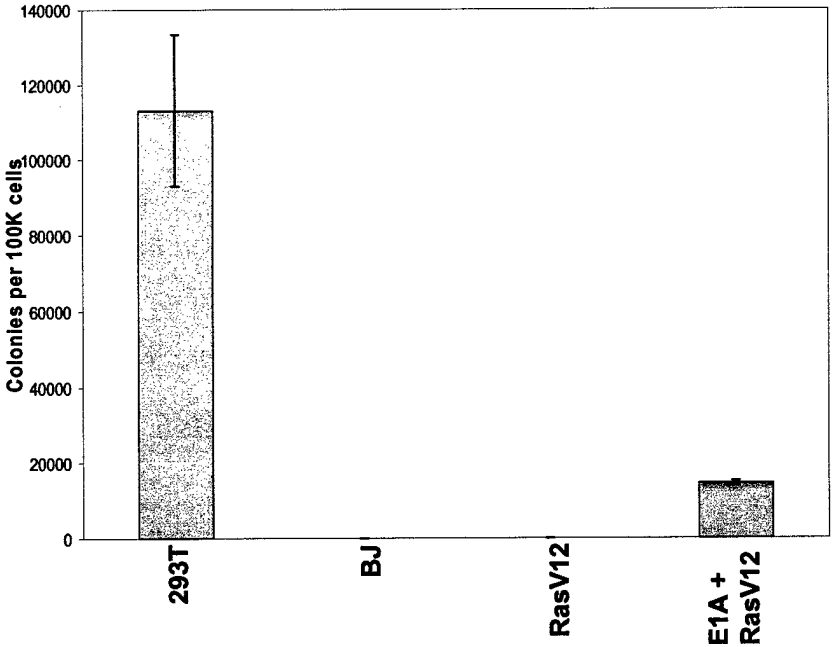
A.



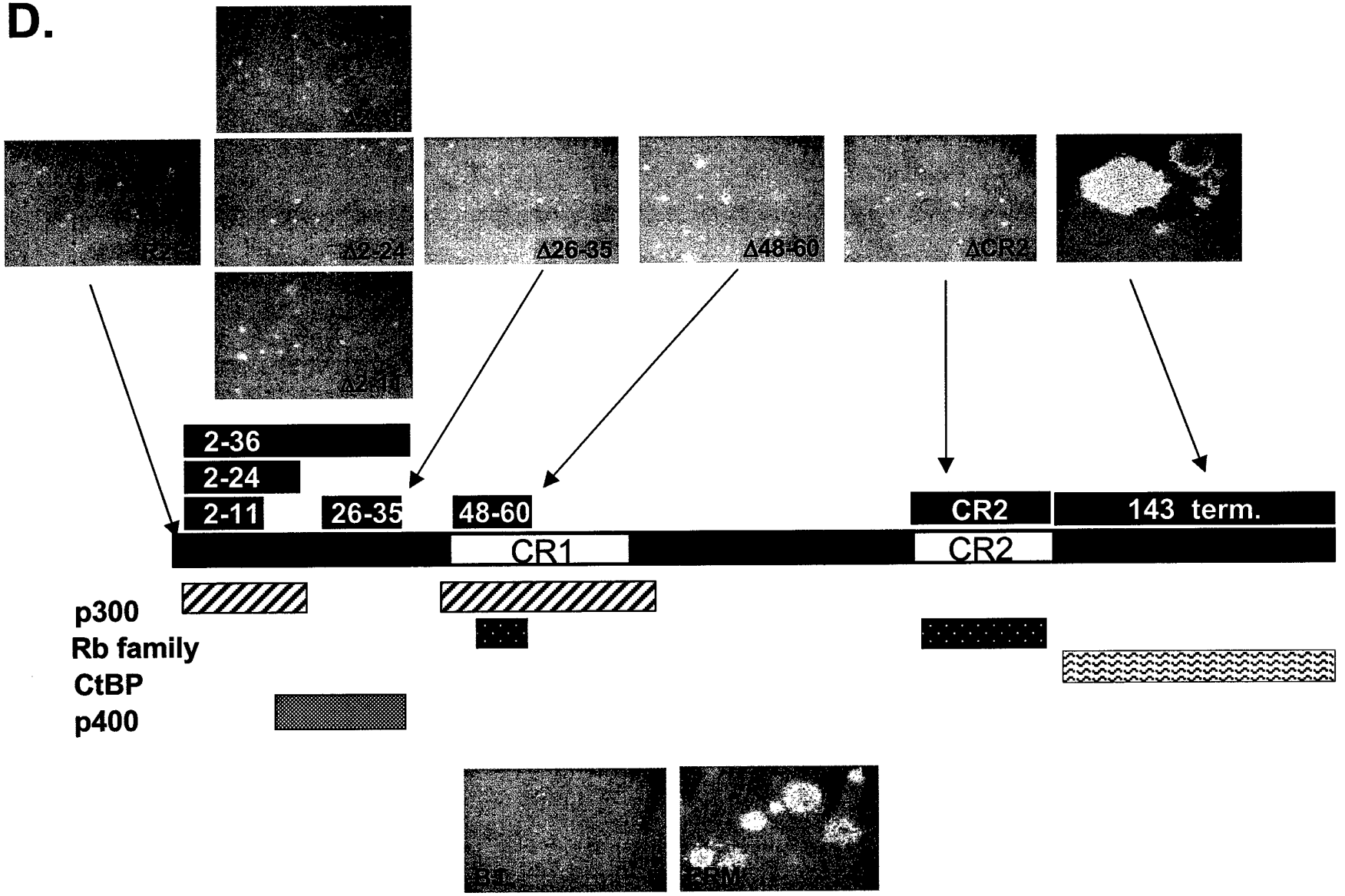
B.



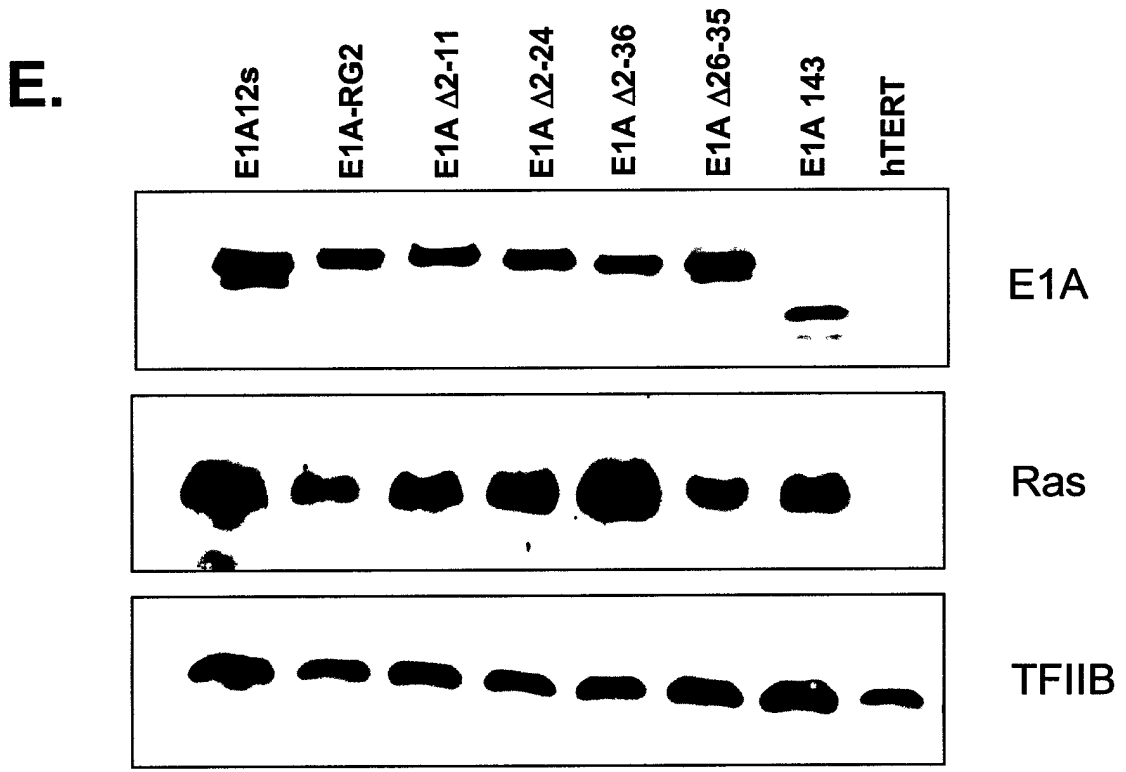
C.



Seger et al., Figure 1D



Seger et al., Figure 1E



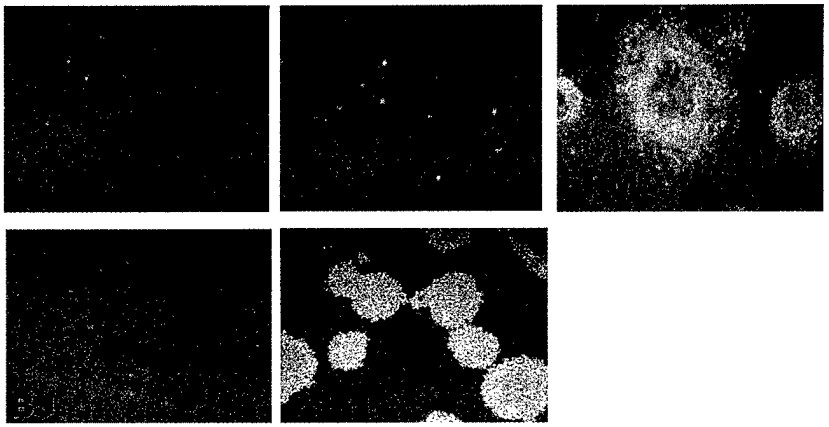
Seger et al., Figure 1F

F.

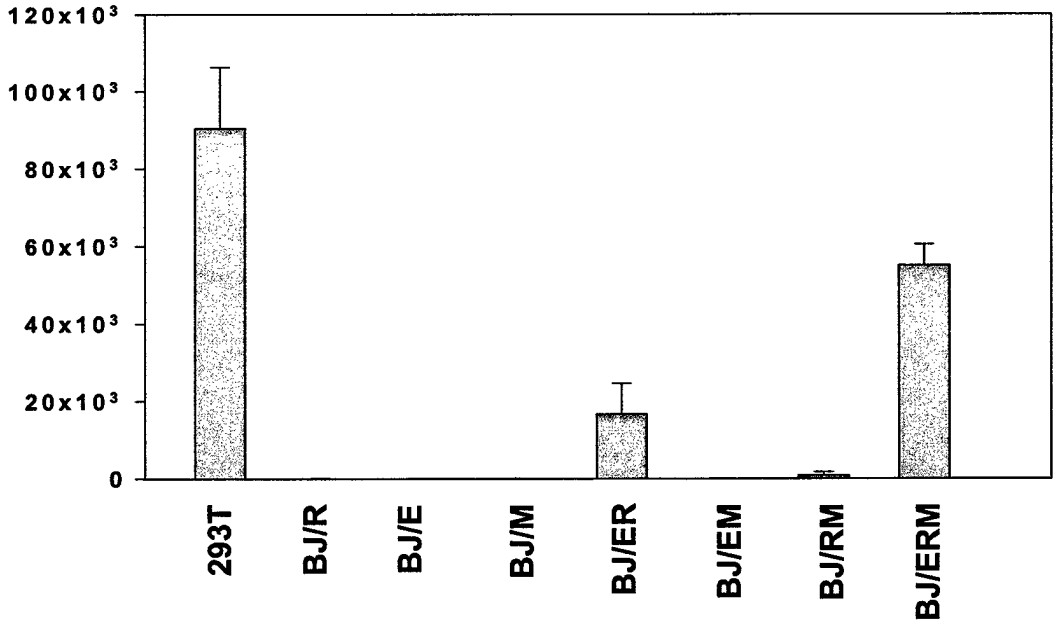
Tumor Formation in Immunodeficient Mice: E1A + Ha-RasV12	
Cell Line	**Number Tumors/Number Injections**
Non-Irradiated Nudes:	
293T	8/8
BJ P18	0/10
BJ/E1A + Ha-RasV12 (serial)	1*/8
BJ/E1A + Ha-RasV12 (coinfection)	0/10
Irradiated Nudes:	
293T	2/2
BJ P18	0/2
BJ/E1A + Ha-RasV12 (coinfection)	0/4
SCID-beige:	
293T	2/2
BJ P18	0/2
BJ/E1A + Ha-RasV12 (serial)	0/4
BJ/E1A + Ha-RasV12 (coinfection)	0/6

Seger et al., Figure 2A and 2B

A.



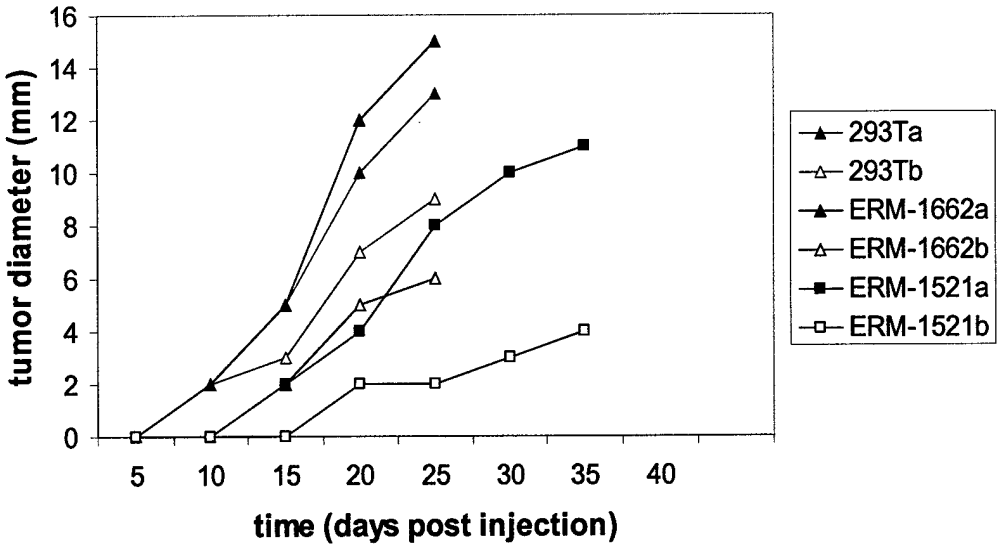
B.



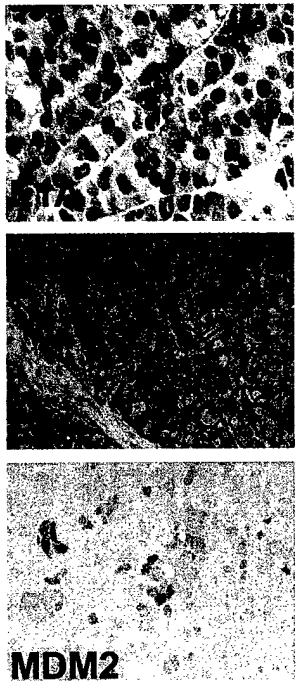
Seger et al., Figure 3A and 3B

A.

B.



C.

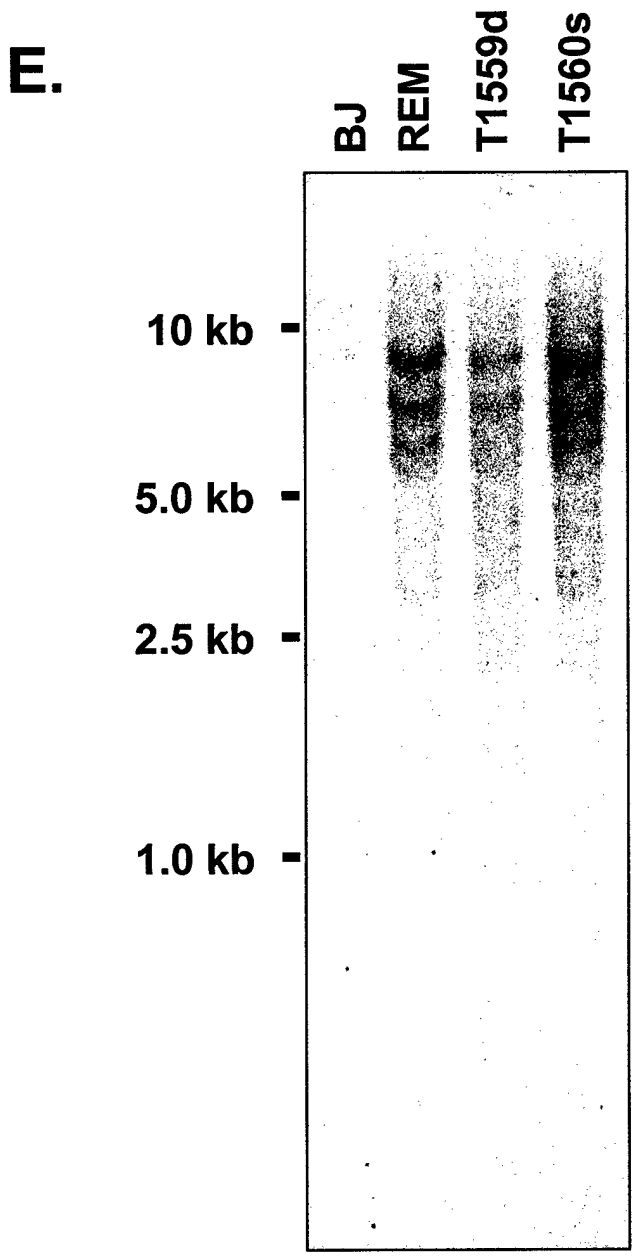


Seger et al., Figure 3D

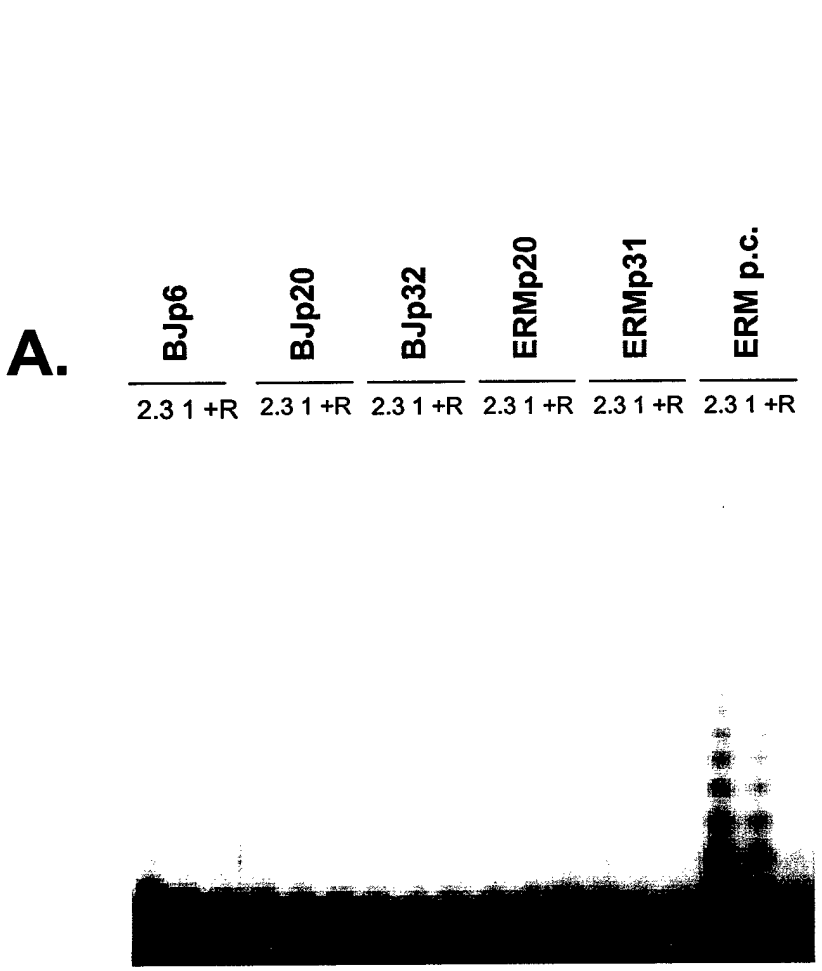
D. Tumor Formation in Immunodeficient Mice: E1A + MDM2 + Ha-RasV12

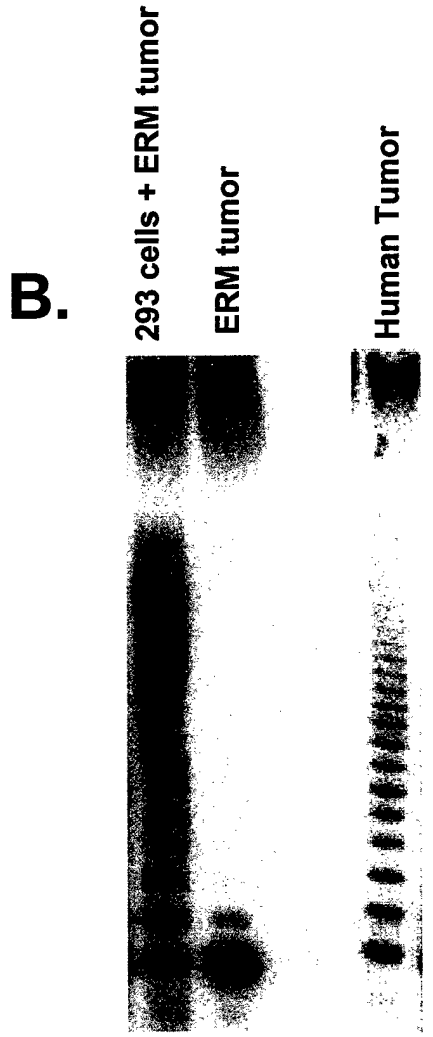
Cell Line	Number Tumors/Number Injected
293T	6/6
BJ	0/10
BJ/E1A + Ha-RasV12	0/6
BJ/E1A + MDM2	0/5
BJ/E1A + MDM2 + Ha-RasV12	34/48
BJ/E1A-143 + MDM2 + Ha-RasV12	6/6

Seger et al., Figure 3E

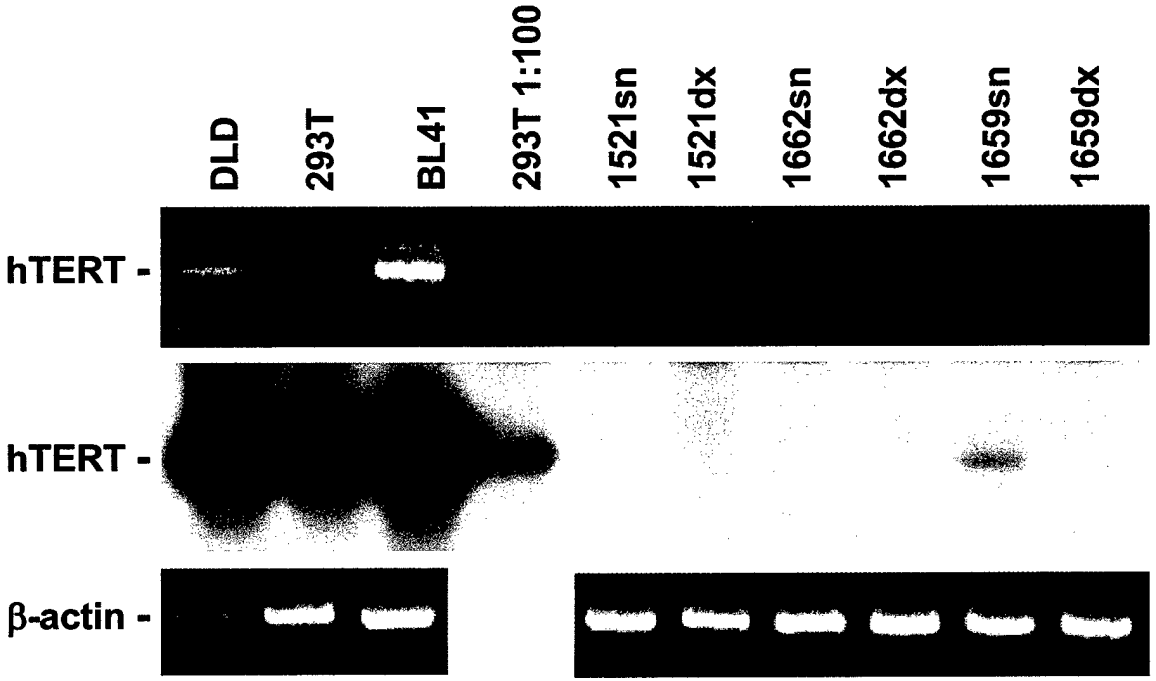


Seger et al., Figure 4A and 4B



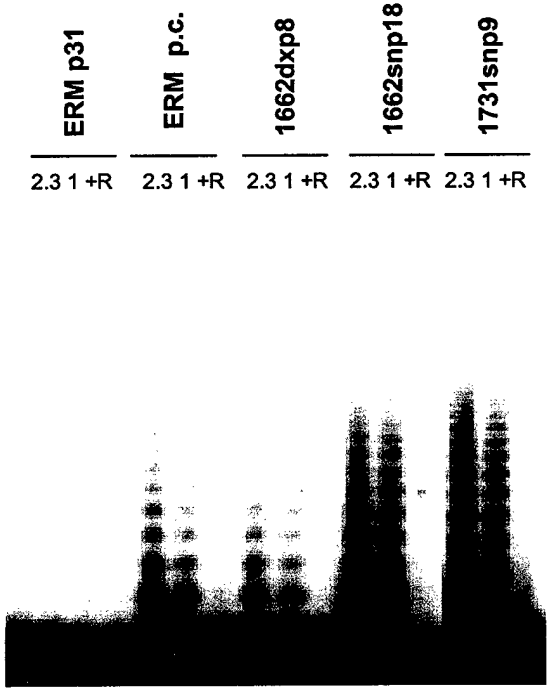


Seger et al., Figure 4C

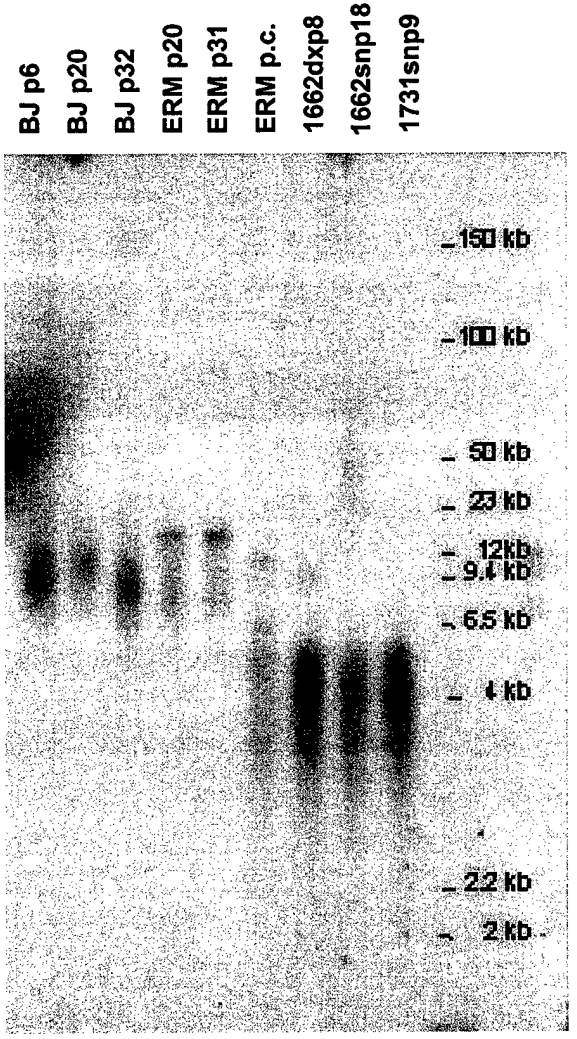


Seger et al., Figures 5A and 5B

A.

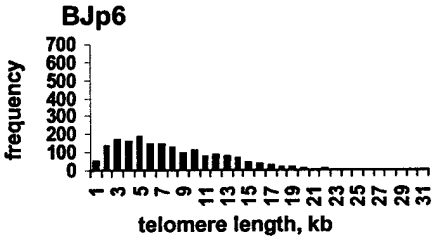


B.

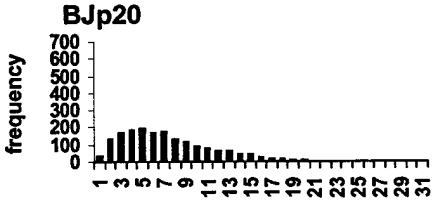


Seger et al., Figure 5C

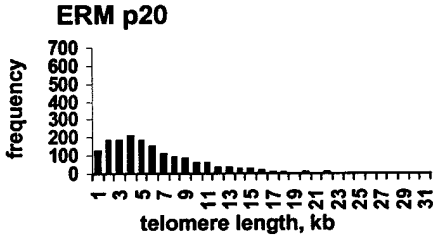
C.



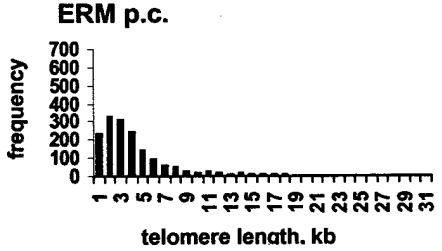
BJp6
p = 6.8 ± 5.0
q = 6.7 ± 4.4
t = 6.8 ± 4.7
% undetectable telomeres = 3.0
total telomeres=1780



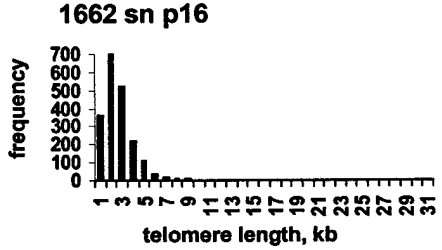
BJp20
p = 6.8 ± 4.5
q = 5.8 ± 4.1
t = 6.4 ± 4.3
% undetectable telomeres= 2.1
total telomeres =1772



ERM p20
p = 5.2 ± 4.2
q = 4.9 ± 4.0
t = 5.0 ± 4.1
% undetectable telomeres= 7.8
total telomeres =1636



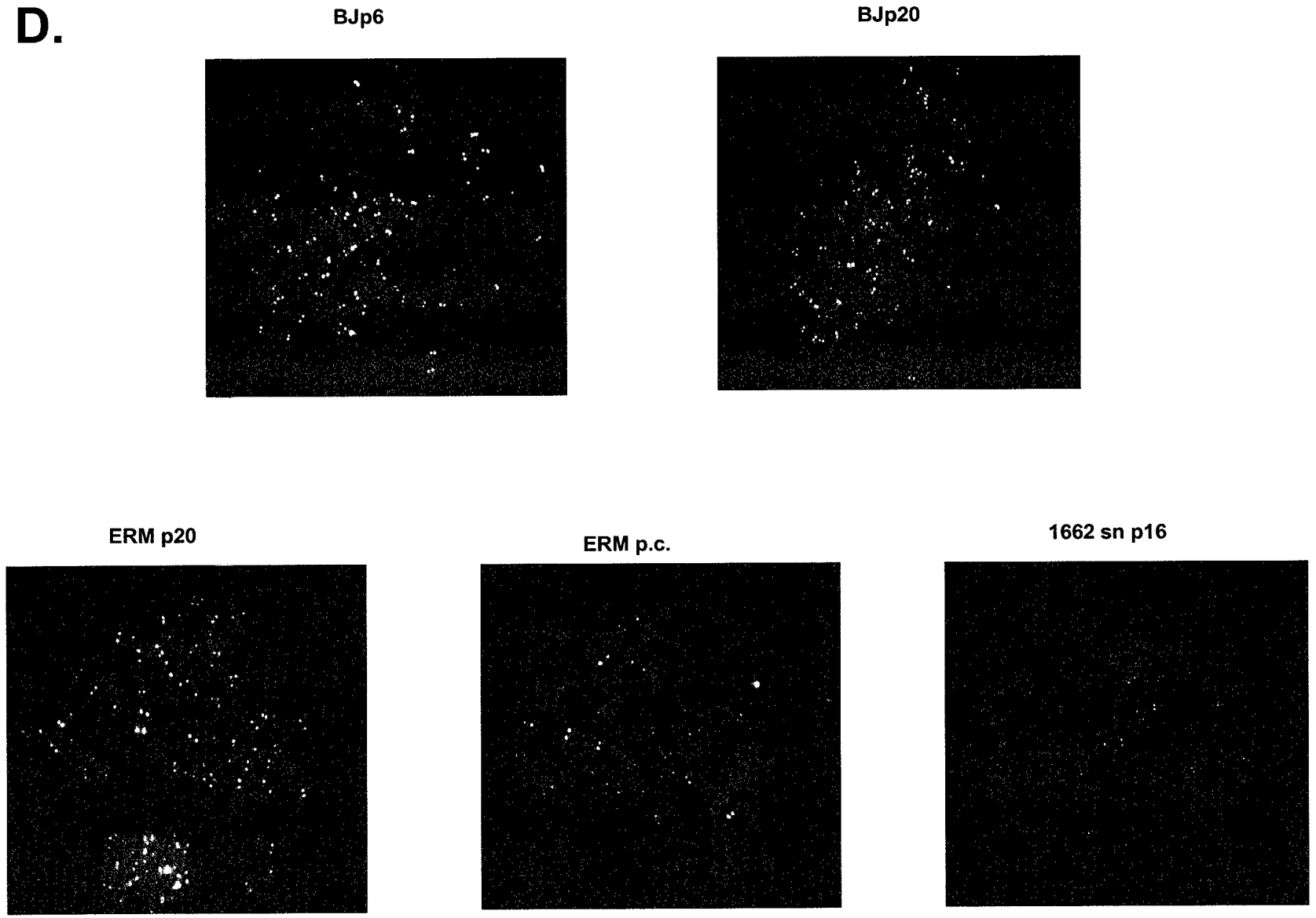
ERM p.c.
p = 3.3 ± 3.4
q = 2.9 ± 3.0
t = 3.1 ± 3.2
% undetectable telomeres= 14.9
total telomeres =1600



1662 sn p16
p = 1.7 ± 1.4
q = 1.6± 1.3
t = 1.6 ± 1.3
% undetectable telomeres= 18.4
total telomeres =1980

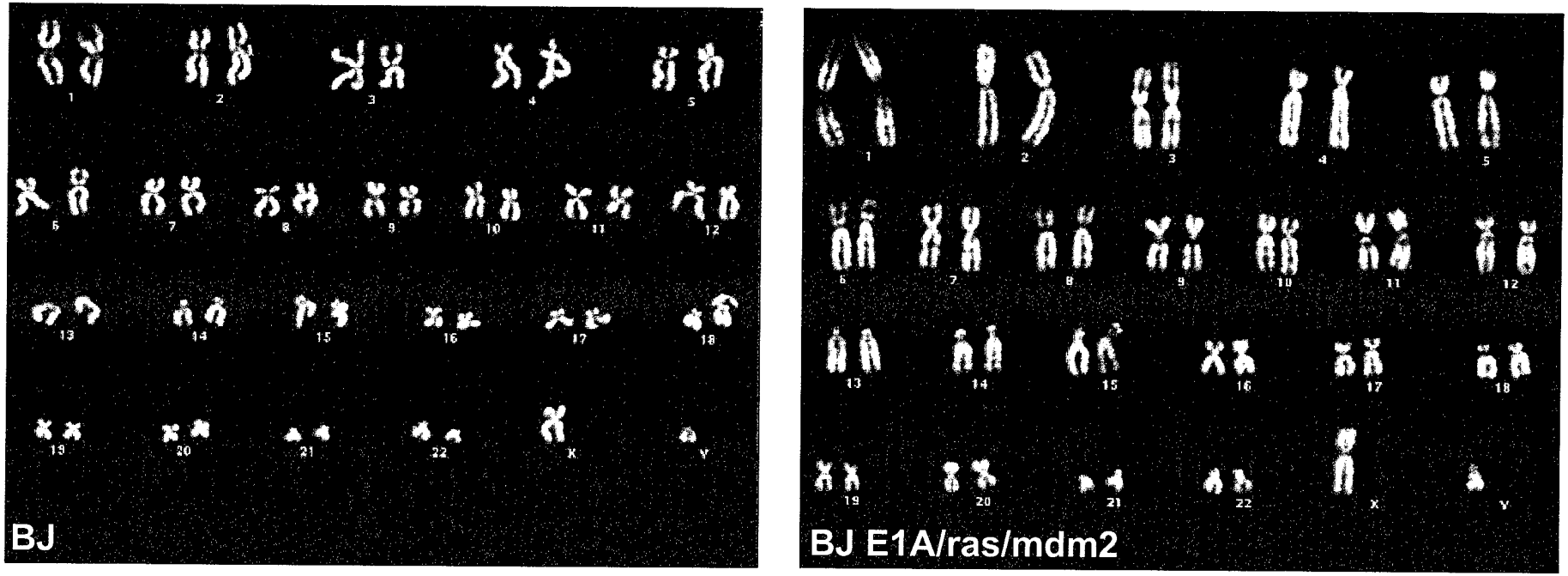
Seger et al., Figure 5D

D.



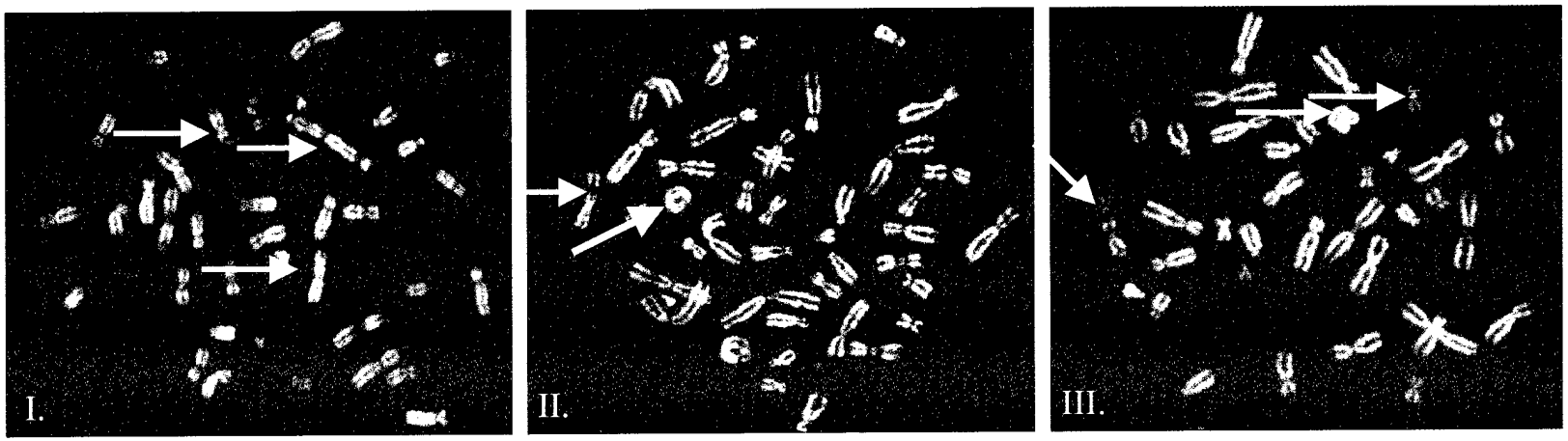
Seger et al., Figure 6A

A.



Seger et al., Figure 6B

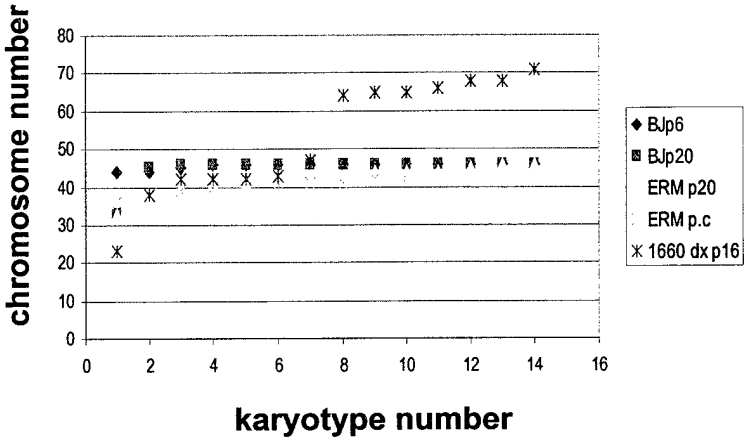
B.



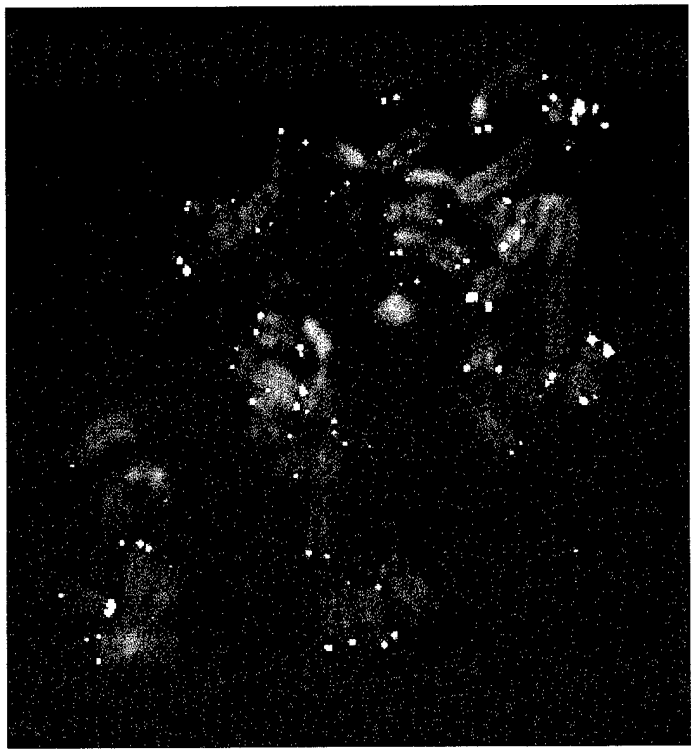
Seger et al., Figure 6C

C.

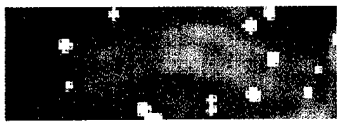
	FRAGM.	DICENT.	TRICENT.	TRANSL.
BJ p6	0%	0%	0%	0%
BJ p20	0%	0%	0%	0%
ERM p20	0.16%	0.16%	0%	0%
ERM p.c.	2%	0.75%	0.5%	1.5%
1660 dx p16	0.67%	0.13%	0%	0%



ERM p.c.

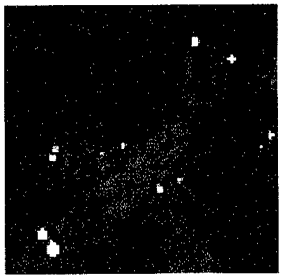


TRANS.



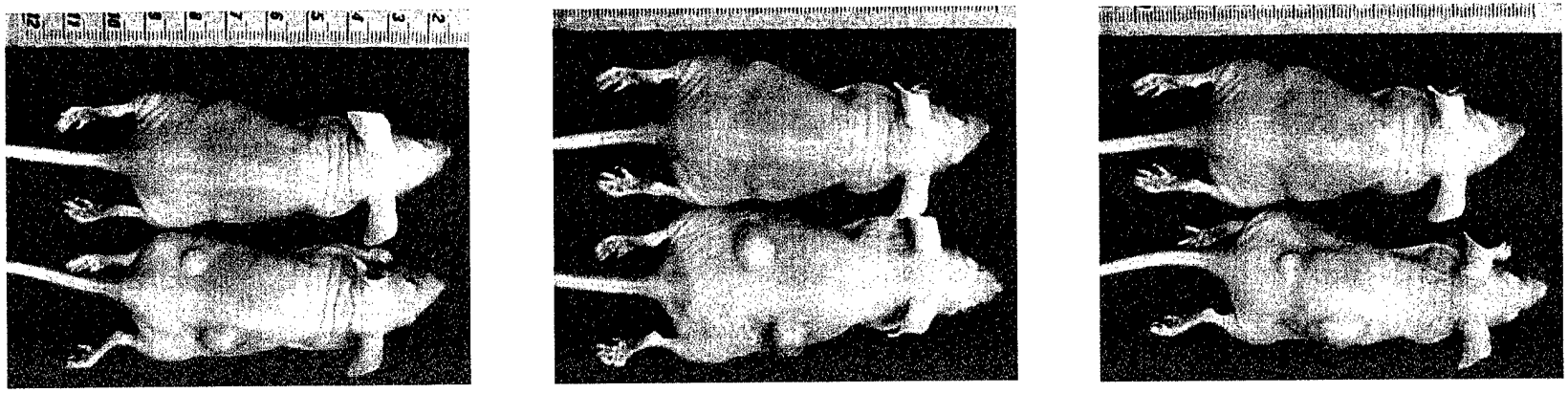
TRICENT.

DICENT.



Seger et al., Figure 7A and 7B

A.



BJ HSF43 WI-38

B.

Tumor Formation in Immunodeficient Mice: E1A + MDM2 + Ha-RasV12

Cell Line	Number Tumors/Number Injected
BJ/ERM	12/12
HSF43/ERM	2/8
WI-38/ERM	7/8
Detroit551/ERM	7/7

Seger et al., Figure 7C