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Using a combination of positional and functional cloning, we have mapped SEN 16 to a 100Kb region at 16q24.3. Intact, normal human chromosome 16, when transferred into immortal human (MCF& SKBR-3) and rat (LA7 & NMU) mammary tumor cells by microcell mediated chromosome transfer (MMCT) restored senescence to these cells. Moreover, transfer of a fragment of chromosome 16 containing 16q23-qter also induced senescence to these tumor cells. Continuous culture of the senescent cells in selection media gave rise to revertant cells which have parental cell growth phenotype and have lost the region of the introduced chromosome that harbors the senescence gene. Analysis of these revertant clones with previously mapped chromosome 16-specific markers placed the senescence gene within a 3-7 cM region at 16q24.3. Three overlapping Yeast-Artificial-Chromosome (YAC) clones spanning this region were obtained. Transfer of one of these YACs, 792E1 (360 Kb), restored senescence in MCF7 and LA7 cells while another YAC containing a portion of chromosome 6 did not alter the growth characteristics of these cells. To further map SEN16, a partial Bacterial-Artificial-Chromosome (BAC) contig was created spanning the YAC 792E1. Experiments are in progress to identify a BAC that harbors SEN16 and to further clone and characterize this gene.

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Deepthi E. Reddy 7/20/99

PI - Signature Date

TABLE OF CONTENTS

1. Front Cover	Page 1
2. Report Documentation Page (SF 298)	Page 2
3. Foreword	Page 3
4. Table of Contents	Page 4
5. Introduction	Pages 5-6
6. Body	Pages 6-8
7. Conclusions	Pages 8-9
8. References	Pages 10-11
9. Figures 1 and 2	Pages 12-13
10. Key Research Accomplishments	Page 14
11. List Of Reportable Outcomes	Page 15
12. Manuscript Addendum	Page 16

ANNUAL REPORT

INTRODUCTION

Normal mammalian cells exhibit a limited proliferative potential (1). At the end of their replicative span, these cells senesce, becoming enlarged, vacuolated and flattened. These cells are metabolically active but are in a state of irreversible G1 arrest and do not divide (2-4). Cellular senescence is a genetically programmed event which is controlled by genes whose collective effect manifests at the end of the cell's life span. In contrast to normal cells, breast cancer and other tumor cells can multiply indefinitely, having escaped senescence as a result of alterations of critical genes (5,6,7). Escape from senescence thus represents an important step in tumor progression. Since senescence genes are involved in negative regulation of cell growth, they are regarded as a class of tumor suppressor genes. The main focus of this project is identification, localization and high resolution mapping of the gene(s) involved in the restoration of senescence to immortal breast tumor cells.

Cytogenetic and loss of heterozygosity (LOH) studies on breast tumors suggest the presence of tumor suppressor genes on human chromosomes 1, 3, 6, 11, 13, 16, 17, 19 and 20 (8,9,10). Increased frequency of LOH and chromosomal deletions on the long arm of human chromosome 16 at 16q22, 16q24 and 16q24-qter has been reported in breast cancer, prostate cancer and hepatocellular carcinoma (11-21). These data suggest an important role for a gene(s) on chromosome 16 during the conversion of normal breast epithelium into a cancerous state.

PREVIOUS WORK

To determine the effect of the introduction of normal human chromosome 16 into breast cancer cells, intact normal human chromosome 16 was introduced into human and rat mammary carcinoma cells by means of microcell mediated chromosome transfer method (MMCT). Human breast cancer cell lines SKBR-3 and MCF7 and rat mammary tumor cell lines NMU and LA7 were used in these experiments. Chromosome transfer clones showed morphological and growth characteristics typical to senescent cells. Continuous cultivation of the senescent cells in selection media gave rise to revertant clones which are morphologically similar to and have the same growth rate as the immortal parent cells. These revertant clones were shown to have lost the region of the introduced human DNA that was capable of restoring senescence to the tumor cells. PCR analysis of 5 human revertants for the presence of previously mapped polymorphic chromosome 16 specific markers localized the senescence gene to the long arm of chromosome 16. Transfer of human chromosome 13 into MCF7 and NMU and transfer of human chromosome 7 into LA7 did not restore senescence to these cells.

A sub-monochromosomal hybrid library developed in Dr. Athwal's lab consists of mouse A9 cells that contain fragments of human chromosome 16 tagged with the *gpt* selectable marker. Nine hybrid clones containing fragments of human chromosome 16 were analyzed cytogenetically and by PCR using previously mapped chromosome 16 markers to determine the size and position of the fragment on chromosome 16. Two fragments of chromosome 16 containing regions 16q22-qter and 16q23-qter were selected for further chromosome transfer studies.

Introduction of these fragments of chromosome 16, containing either the region 16q22-qter or 16q23-qter also induced senescence in both the human and rat breast tumor cells.The same phenotype was observed in several independent experiments. Furthermore, these fragments induced senescence in ovarian cancer cells and in SV40 transformed human and mouse fibroblast cells. These results confirm the involvement of 16q22-qter in restoration of senescence to immortal tumor cells by functional complementation.

Initial analysis of five rat (mammary tumor) revertant clones with fifteen chromosome 16 specific markers had localized the senescence gene to 16q24.2-16q24.3. Further analysis with 30 rat revertants using an additional 60 markers from 16q23-qter was done. A consensus deletion map was derived from the presence or the absence of these markers in the revertant clones. This consensus deletion region contains 21 markers. Based on information from genetic and physical maps of these markers available from various databases, we mapped the region of the senescence gene (SEN 16) to a 3-7 cM region at 16q24.3.

The remaining fragment of chromosome 16q23-qter present in a rat revertant clone of LA7 which lost the region of the chromosome that harbored the senescence gene, was transferred into the human breast tumor cell line, MCF7. This fragment of human DNA did not restore senescence to the human breast tumor cells. These results indicate that the same region of chromosome 16, perhaps the same gene at 16q24.3, restores senescence in both rat and human immortal tumor cells.

To further localize SEN16, a partial YAC contig was assembled corresponding to the 3-7 cM region at 16q24.3 and analyzed for marker content. A 360 kb YAC from this contig, 792t2, was found to restore senescence to rat and human mammary tumor cells, and, to rat ovarian tumor cells.

BODY

EXPERIMENTAL METHODS

Cell Lines and Growth Conditions

The cell lines used are human breast tumor cell line, MCF7, (ATCC); rat mammary tumor cell line LA7 (22); and, mouse immortal epithelial cell line, A9 (ATCC). All cultured were maintained in DF12 medium supplemented with 10-15% FBS at 37^{0}C in 7.5% CO_2 incubators. YAC transfer clones are grown in 400 µg/ml of G418 containing media.

Growth of BAC Clones

All BAC clones were grown in LB medium (Sigma) containing 20 µg/ml chloramphenicol (Sigma). Retrofitted BAC clones were grown in LB medium containing 20 µg/ml chloramphenicol and 30 µg/ml of kanamycin (Sigma).

Isolation of BAC DNA (mini-prep)

To isolate BAC DNA from small culture volumes, a standard mini-prep protocol was used (protocol from Research Genetics, Huntsville, AL). Briefly, a BAC clone was grown overnight at 37 °C in 1.5 ml LB medium containing 20 µg/ml of chloramphenicol (and 30 µg/ml of kanamycin where necessary). Cells were harvested at high speed and the pellet was resuspended in 100 µl of solution I containing 50 mM glucose, 20 mM Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0. 200 µl of freshly prepared solution II containing 0.2 N NaOH, 1% SDS was then added, the contents were mixed and the tube was placed on ice for 1-2 minutes. Next, 150 µl of potassium acetate (contains 3 M potassium and 5 M acetate) was added to the reaction mixture. The contents were spun down at full speed for 6 minutes in a microcentrifuge at room temperature. The supernatant was then transferred into a fresh tube and the DNA was precipitated with 1 ml of ethanol. The pellet was then washed several times in 70% ethanol, dried and dissolved in 20 µl of TE buffer.

Isolation of BAC DNA (maxi-prep)

To isolate large quantities of BAC DNA, the QIAGEN Maxi protocol was followed. The BAC clone was first grown in 5 ml LB medium containing the appropriate antibiotic and 0.5 ml of this preculture was inoculated into 100 ml LB medium containing the antibiotic(s). The culture was grown at 37 °C for 14 hours with vigorous shaking. Cells were then harvested by centrifugation in 50 ml tubes and resuspended in 10 ml of buffer P1 containing 100 µg/ml RNase A, 50 mM Tris-Cl, pH 8.0, 10 mM EDTA. Next 10 ml of lysis buffer P2 containing 200 mM NaOH and 1% SDS was added. The contents were mixed gently and incubated at room temperature for 5 minutes. Next, 10 ml of chilled neutralization buffer P3 containing 3 M potassium acetate, pH 5.5 was added. The contents were mixed gently, incubated on ice for 15 minutes, and spun down at 20,000 x g for 30 minutes at 4 °C. The supernatant was removed into fresh tubes and the solution was re-centrifuged at 20,000 x g for another 15 minutes at 4 °C. The supernatant was then applied to a QIAGEN-tip 100 equilibrated with 4 ml of QBT buffer (750 mM NaCl, 50 mM MOPS, pH 7.4, 15% isopropanol and 0.15% Triton-X 100) and the column was allowed to empty by gravity. The tip was then washed with 2 x 10 ml QC wash buffer (1 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol). The DNA was then eluted with 5 aliquots of 1 ml QF elution buffer (1.25 M NaCl, 50 mM Tris-Cl, pH 8.5 and 15% isopropanol), prewarmed to 65 °C. The BAC DNA was then precipitated by adding 3.5 ml of room-temperature isopropanol to the eluted DNA and mixing gently. The contents were centrifuged at 15000 x g for 30 minutes at 4 °C. The pellet was washed in 2 ml of 70% ethanol, air-dried and redissolved in TE, pH 8.0. Yield of BAC DNA from this prep was typically 20-40 µg from a 100 ml BAC culture.

Restriction Enzyme Digestion of BAC DNA

BAC inserts wee excised from the vector using the restriction enzyme Not I (New England Biolabs). 1 µg of BAC DNA was digested with Not I restriction enzyme in NEBuffer 3 supplemented with 100 µg/ml BSA. Complete Not I digestion released the genomic insert and was visualized by separating the digested fragments by PFGE.

For comparison of the restriction enzyme digestion patterns between two BAC clones, DNA digested with Hind III restriction enzyme in buffer E at 37 °C and separated on a 1% agarose gel containing ethidium bromide.

Pulsed Field Gel Electrophoresis

PFGE of BAC DNA was performed with the CHEF Mapper system from Bio-Rad. The digested DNA was separated on a 1% SeaKem GTG agarose gel using 0.5X standard TBE buffer at 14 °C. The MidRange II PFG Marker (NEB) was used as the DNA size standard. The gels were run at 200V (6 V/cm approximately), with a pulse angle of 120° and a pulse time of 5-15 seconds. Gels were stained in 0.5 X TBE buffer with 0.2 µg/ml ethidium bromide for 45 minutes or more with gentle shaking and photographed on a UV transilluminator.

RESULTS AND DISCUSSION

Screening of a BAC Library for Clones Spanning the Consensus Deletion Region

Two approaches were used to identify BACs carrying markers from the consensus deletion region at 16q24.3. A PCR based BAC library, obtained from Research Genetics (Huntsville) was screened using PCR primers for markers in the consensus deletion region. Some of the markers analyzed did not yield any positive clones in this library screen. For most of these markers, BAC

clones were identified using information from the Caltech database. This eliminated the time consuming process of screening other libraries for identification of positive BAC clones for the non-informative markers.

The primer pairs used to screen the BAC library are D16S413, 498, 520, 2750, 2801, 3037, 3048, 3061, 3063, 3077, WI-15838, 12410 and EST00889. No positive BAC clones were obtained with the primers D16S498, 520, 2801, 3077, WI-15838 and EST00889. However, the markers D16S413, 2750, 3037, 3048, 3061, 3063 and WI-12410, were informative in obtaining BAC addresses. The corresponding BACs for these markers were purchased from Research Genetics and marker content verified by PCR.

The Human Genome Project at Caltech is involved in isolation and sequencing of YAC, P1 and BAC clones from chromosome 16. Search of their database (www.tree.caltech.edu) identified several BAC clones carrying markers from the long arm of chromosome 16. Using this information, BAC clones corresponding to the primer pairs, D16S305, 413, 449, 476, 486, 498, 2727, 2733, 2772, 2801 and 2866, were identified. All of these BAC clones were purchased from Research Genetics.

DNA from each of the BAC clones described above was analyzed to confirm the presence of the markers that were used to identify the BAC during screening. Next, these clones were analyzed by PFGE to determine the size of the human DNA inserts and to identify internal Not I sites.

BAC clones were assembled in a contig corresponding to the retrofitted YAC 792t2 which was shown in earlier studies to induce senescence in breast tumor cell lines (Figure 1). In addition, partial contigs of the consensus deletion region at 16q24.3 were created using marker content information. The BAC clones present in these contigs are 440F1 (D16S3037, 3061), 411M22 (D16S3063, 3048), 411M24 (D16S3063, 3048), 346J21 (D16S3063, 3048, WI-12410, 15838), 344A17 (D16S3048, WI-12410, 15838, TIGRA001Y26, SHGC3238, StSG4762, SGC36958), 344B17 (D16S3048, WI-12410, 15838, TIGRA001Y26, SHGC3238, StSG4762, SGC36958), 208I2 (D16S2750), 276J15 (D16S2750), 277H16 (D16S2750), 500H4 (D16S413), 500P7 (D16S413), 576G12 (D16S413), 339P10 (D16S413), 143F5 (D16S498), 214B1 (D16S486), 264G9 (D16S2801), 924B9 (D16S476), 924B1 (D16S476), 923C6 (D16S476), 15E10 (2772), 343H9 (EST00889, D16S413), 128B5 (D16S413, 305), 196H2 (D16S305), 318G12 (D16S305, 449), 318H1 (D16S305, 449), 158B12 (D16S2751), 412B8 (D16S2866), 351H9 (D16S2733) and 696H9 (D16S2727). No BAC clones containing the markers form the consensus deletion, D16S520, 3077, WI- 3661 and 16080 were identified. Based on some of the above information, markers were arranged to construct a map of the region 16q24.3. These assignments are reflected in Figure 19.

Evaluation of a Contig Spanning the YAC 792t2

BACs 344A17, 346J21, 411M24 and 411M22 contain most of the markers present on the YAC 792t2, that was shown to restore senescence to immortal mammary tumor cell lines. Since they were found to be overlapping by marker content, further analysis was done to determine the extent of this overlap by Southern hybridization. The BAC 344A17 was used as a probe since it contains the largest human DNA insert by PFGE and PCR analysis. This BAC contains all the markers present on BAC 346J21. Southern analysis showed that this BAC, 344A17, hybridized only with the BACs from the SEN16 locus and not with the other BACs from the neighboring region (Figure 2).

CONCLUSIONS

Using an unique functional-positional strategy devised to identify and clone senescence genes, my research implicated a role for human chromosome 16 in restoring cellular senescence to immortal breast and ovarian tumor cells. The gene(s) inducing senescence was further localized to

chromosome 16q24.3 by transferring fragments of the long arm of human chromosome 16 containing the selectable marker, *gpt*, and analyzing the microcell hybrids for human chromosome 16 specific markers. A YAC contig was assembled across the 3-7 cM consensus deletion region. A 360 kb YAC from this deleted region was further shown to induce senescence to immortal breast and ovarian tumor cells.

This report describes efforts to further map the senescence gene, SEN16. A BAC contig was created across the consensus deletion region and the 360 kB YAC, 792t2. Five BACs, 344A17, 344B17, 346J21, 411M24 and 411M24 were found to span the YAC 792t2. The senescence gene is expected to be present on one or more of these BACs. Experiments are in progress to retrofit these BACs with a selectable marker, *neo*, and to identify a BAC(s) that restore senescence to immortal breast tumor cell lines.

Introduction of SEN16 complements a common, if not universal defect in human and rat mammary tumor cells, as well as in other tumor cells. Elucidation of the role of SEN16 in immortalization and malignant transformation await cloning and sequencing of this gene.

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involved in allelic imbalance on chromosome arm 16q in breast cancer. Genes Chrom. Cancer 9: 101-107

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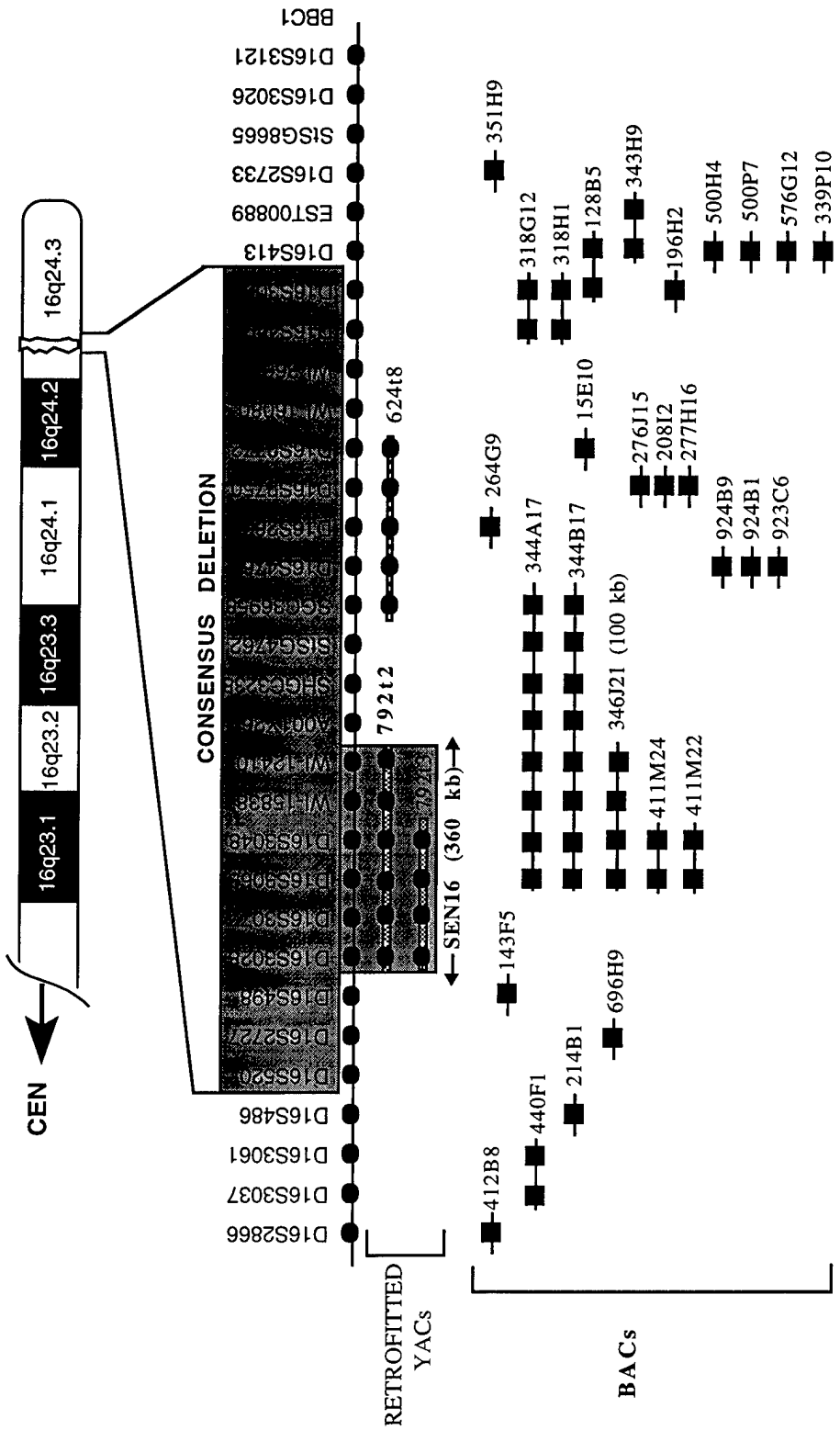

Figure 1. BAC Contig of the Consensus Deletion Region. Markers present on the retrofitted YAC and BACs are shown. The SEN16 locus is indicated by the shaded region. The YAC 792t2, restores senescence to immortal mammary tumor cell lines and is shown in bold. The BACs spanning this YAC are also shown.

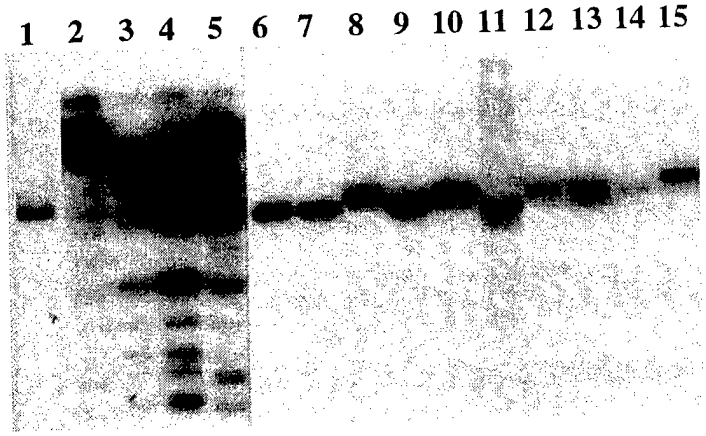

Figure 2. Southern Analysis of BACs Spanning the Consensus Deletion. Southern hybridization of HindIII digested BACs with BAC 344A17 (lane 5) as probe demonstrates overlap with BACs corresponding to YAC 792t2: 411M24 (lane 2), 411M22 (lane 3) and 346J21 (lane 4). When hybridized to other HindIII digested BACs from the consensus deletion region, no DNA overlap was seen between this BAC and BACs 143F5 (lane 6), 214B1 (lane 7), 208I2 (lane 8), 264G9 (lane 9), 924B9 (lane 10), 15E10 (lane 11), 128B5(lane 12), 196H2(lane 13), 318G12(lane 14), 696H9 (lane 15). The vector (7.4 kb), pBeloBAC 11 is shown in lane 1. BACs are from different libraries and therefore contain vector bands of slightly different sizes.

KEY RESEARCH ACCOMPLISHMENTS

- Transfer of intact, normal human chromosome 16 restores senescence to immortal human and rat mammary tumor cells, and , to immortal rat ovarian tumor cell lines
- Transfer of normal, fragments of human chromosome 16q also restore senescence to immortal human and rat mammary tumor cells, and , to immortal rat ovarian tumor cell lines. Senescence gene, SEN 16, is thus localized to 16q23-qter.
- PCR analysis of rat revertant clones maps SEN 16 to a 3-7 cM region at 16q24.3
- A partial YAC contig spanning the 3-7 cM consensus deletion region was assembled. YACs were retrofitted with a selectable marker, neo. Transfer of YACs into mammalian cells further localized SEN16 to a 360 kb YAC, 792t2.
- A BAC contig was assembled spanning the consensus deletion region and he 360 kb YAC.
- SEN 16 mapped to a small genomic region (<200 kb) at 16q24.3 using a unique functional-positional cloning approach.

LIST OF REPORTABLE OUTCOMES

- **MANUSCRIPT**

  Title: Identification of a Gene at 16q24.3 that Restores Cellular Senescence in Immortal Mammary Tumor Cells

  Authors: Deepthi E. Reddy, Arbansjit K. Sandhu, Jon K. DeRiel, Raghbir S. Athwal and Gursurinder P. Kaur

  Fels Institute for Cancer Research, Temple University School of Medicine, Philadelphia, PA 19140

  Journal: Oncogene, in press

- **Degrees Obtained:** Doctor of Philosophy
  Candidate: Deepthi E. Reddy

- **Employment Opportunities:** The candidate, Deepthi E. Reddy has applied for post-doctoral fellowship positions based on experiences/training supported by this award

Identification of a Gene at 16q24.3 that Restores Cellular Senescence in Immortal Mammary Tumor Cells

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Running Title: SEN16, a Cell Senescence Gene at 16q24.3

Key Words: Cell Senescence, Gene mapping, Immortalization, Breast Cancer, Chromosome transfer,

ABSTRACT

We have mapped a cellular senescence gene, SEN16, within a genetic distance of 3-7cM, at 16q24.3. Microcell mediated transfer of a normal human chromosome 16, 16q22-qter or 16q23-qter restored cellular senescence in four immortal cell lines, derived from human and rat mammary tumors. The resumption of indefinite cell proliferation, concordant with the segregation of the donor chromosome, confirmed the presence of a senescence gene at 16q23-qter. While microcell hybrids were maintained in selection medium to retain the donor chromosome, sporadic immortal revertant clones arose among senescent cells. Reversion to immortal growth could occur due to inactivation of the senescence gene either by a mutation or a deletion. The analysis for chromosome 16 specific DNA markers, in revertant clones of senescent microcell hybrids, revealed a consensus deletion, spanning a genetic interval of approximately 3-7cM at 16q24.3.

Introduction

Normal diploid mammalian cells undergo replicative senescence after a finite number of population doublings (Hayflick and Moorehead, 1961; Hayflick, 1965). In contrast, cells cultured from many tumors can either proliferate indefinitely or have an increased proliferative lifespan (Stamps et al., 1992). Senescent cells are incapable of DNA replication but remain metabolically active for an extended period of time and are resistant to apoptosis (Goldstein, 1990; Wang, 1995). Cellular senescence is a genetically programmed process, expressed as a dominant phenotype over indefinite proliferation in hybrids between normal and immortal cells (Bunn and Tarrant, 1980; Pereira-Smith and Smith, 1983). By somatic cell hybrid analysis among a large number of immortal cell lines of diverse origin, four complementation groups have been identified for indefinite proliferation (Pereira-Smith and Smith, 1988).

Spontaneous immortalization can occur in cultured rodent cells (Barrett and Ts'o, 1978) but has not been observed in human cells. Transformation of normal human cells with oncogenic DNA viruses extends the proliferative lifespan but such cells eventually enter a state of irreversible growth arrest (Gotoh et al., 1979; Neufield et al. 1987). However, human cells transformed with oncogenic DNA viruses can give rise to immortal clones at a low frequency (Huschtscha and Holliday, 1983; Neufield et al., 1987; Shay and Wright, 1989). The immortalization of cells following transformation with DNA viruses has been shown to involve alterations in the cell genome (Neufield et al., 1987; Goolsby et al., 1991; Hubbard-Smith et al., 1992; Steenbergen et al., 1998). Thus, acquisition of indefinite proliferation is a multistep process and requires inactivation of cellular genes in addition to the function of proteins encoded by the viral genome (Shay et al., 1991; Hubbard-Smith et al., 1992).

Cellular senescence has been postulated as a mechanism of tumor suppression, and immortalization appears to be an important step in tumor progression (O'Brien et al., 1986; Sager, 1989; Yeager et al., 1997). Suggestive evidence for the role of senescence in protection against tumorigenesis comes from the comparison of normal and pre-neoplastic immortal mammary epithelial cells, transplanted into cleared mammary fat pads (Daniel et al., 1983). The proliferation of normal mammary cells declines after 5-6 serial passages ending in a state of senescence, whereas pre-neoplastic immortal cells can be passaged indefinitely and eventually give rise to neoplastic growth (reviewed in Medina, 1996; Daniel et al., 1983, Medina et al., 1993). Similarly, in vitro cultures of myoinvasive transitional cell carcinomas (TCC) of bladder give rise to immortal cell lines, while superficial non invasive TCC cells, like normal human uroepithelial cells, undergo senescence after a limited number of population doublings (Yeager et al., 1997). These studies suggest that escape from senescence is essential for neoplastic expansion and may accelerate tumor progression by increasing the opportunity for additional mutations in proliferating cell populations.

Functional complementation by microcell mediated chromosome transfer into tumor cells has been used to identify chromosomes carrying genes which suppress in vitro cell growth (reviewed in Stanbridge, 1992), tumorigenicity and/or metastasis in nude mice (Goyette et al, 1992; Negrini et al., 1994; Welch et al., 1994) or restore cellular senescence (reviewed in Oshimura and Barrett, 1997). Using this approach, putative cell senescence genes have been identified on chromosomes 1, 2, 3, 4, 6, 7, 17, 18 and X (Hensler et al., 1994; Uejima et al., 1995; Rimessi et al, 1994; Ning et al., 1991; Gualandi et al., 1994; Sandhu et al., 1994; Sandhu et al., 1996; Ogata et al., 1993; Yamada et al., 1990; Wang et al, 1992; and our unpublished results). Conceptually these studies imply that a single gene, present on a donor chromosome, confers senescence in recipient cells. Reversion to indefinite proliferation can occur due to

inactivation of this gene by mutation or deletion of the senescence gene (Sandhu et al., 1996). In this paper we report the identification of a gene (SEN16) on chromosome 16 that restores senescence in human and rat mammary tumor cells. We have applied a deletion mapping approach to define the position of the gene within a genetic interval of 3-7cM at 16q24.3.

Results

Chromosome Transfer into Tumor Cell Lines

Normal human chromosomes 7, 13 or 16 as well as chromosome fragments 16q22-qter or 16q23-qter, each tagged with *gpt*, were transferred individually into human and rat mammary tumor cell lines via microcell fusion. Microcell hybrids were recovered in MX selection medium with an average frequency of 1 per 10^6 recipient cells.

Chromosome transfer colonies were maintained in MX medium and examined at regular intervals to assess colony and cell morphology and growth characteristics. Based on these criteria, microcell hybrid clones fell into two distinct classes. 1) Immortal colonies were indistinguishable from the parental tumor cells, which proliferate indefinitely with a doubling time of 15-30h. 2) Senescent colonies consisted of enlarged flattened vacuolated cells, with an initial doubling time of 72-96h that increased progressively until complete growth arrest after 6-8 weeks (Table 1, Fig.1). At this stage, each senescent colony contained between 500 and 2000 cells which remained attached to the surface in a nondividing state for an additional 1-2 months.

Following the transfer of chromosome 16, 16q22-qter or 16q23-qter, a total of 56 independent microcell hybrid colonies were obtained in two human breast tumor cell lines (MCF.7

and SKBR-3), while 99 colonies were recovered in two rat mammary tumor cell lines (NMU and LA7) (Table 1). All microcell hybrid clones carrying chromosome 16, 16q22-qter or 16q23-qter displayed the senescent phenotype (Table 1, Fig. 1). In contrast, the introduction of chromosome 7 into LA7 or chromosome 13 into MCF.7 and NMU cells did not affect the morphology or proliferation potential of the recepient cell lines (Table 1, Fig. 1). These results show that chromosome 16 carries a gene, located in the region 16q23-qter, that induces senescence in human as well as in rat mammary tumor cells.

Analysis for The Presence of Donor Chromosome in Senescent Microcell Hybrids

DNA prepared from senescent chromosome transfer clones was analyzed by PCR for the presence of chromosome 16 specific markers, in parallel with DNA from donor and recipient cells. Since donor chromosomes are tagged with gpt, retention of gpt in all microcell hybrids was confirmed by PCR (Fig.2A). Microcell hybrids of MCF.7 and SKBR-3 cells were examined for the presence of donor chromosome 16 alleles for 15 polymorphic microsatellite $(CA)_n$ repeat markers, while colonies of rat cells were analyzed for 89 markers. A typical example of this type of analysis is presented in figure 2 and a list of the markers is given in Material and Methods. These experiments confirmed that senescent microcell hybrids retained all donor chromosome markers. The presence of the donor chromosome in microcell hybrids of rat cells was also confirmed by cytogenetic analysis by FISH (Fig. 3).

Reversion to Indefinite Proliferation Concordant with the Segregation of Donor Chromosome

If the donor chromosome is indeed responsible for cell senescence in chromosome transfer

clones, its loss should result in reversion to indefinite proliferation. Cells from pre-senescent microcell hybrid colonies were cultured in non-selective medium to permit loss of the donor chromosome by random segregation. Independent immortal segregant clones, which arose in pre-senescent cell populations, were isolated individually for each recipient cell line. Segregant clones were examined for the presence of gpt_ and chromosome 16 specific markers. This analysis revealed that segregant clones no longer contained the donor human chromosome (data not shown). Reversion to immortal growth, concordant with loss of the donor human chromosome, confirmed that the restoration of senescence in tumor cells requires the retention of donor chromosome 16, 16q22-qter or 16q23-qter.

Localization of the Senescence Gene within a 3-7cM Genetic Interval at 16q24.3

While senescent microcell hybrid colonies were maintained in MX medium, fast growing parental type immortal revertant clones appeared spontaneously in senescent cell populations. Such revertants, which retained the gpt tag and most of the donor chromosome, must result from inactivation of the senescence gene through mutation or deletion.We isolated 5 and 16 independent MX revertant clones from microcell hybrids of human and rat cells, respectively, containing chromosome 16, 16q22-qter or 16q23-qter.

To facilitate high resolution mapping of the senescence gene, all MX revertant clones were tested by PCR for markers mapped to the region 16q22-qter. As expected, all revertant clones retained the gpt tag. Of the 16 rat revertant clones, 13 (represented by Rev 1 in figure 4) retained all 89 markers tested but 3 revertants Rev 2, Rev 3 and Rev 4 (Fig.4) each lost a block of contiguous linked markers. Figure 2 shows representative PCR analysis of 4 markers which are deleted in Rev

2, Rev 3 and Rev 4 and figure 4 summarizes the results from 36 markers located at 16q24.2-16q24.3. MX revertants like Rev 1 which retained all the markers may carry point mutations or small deletions in the senescence gene, not detectable with the available set of markers. While the size of the deletion varied among Rev 2, Rev 3 and Rev 4, they all shared a consensus deletion of a set of 21 contiguous markers (Fig. 4).The consensus deletion is flanked by markers D16S486 and D16S413 (Fig. 4). According to the latest integrated map of chromosome 16 in genetic databases, D16S486 and D16S413 are separated by a genetic distance of less than 7cM at 16q24.3 (NCBI database; Doggett et al., 1995; Kozman et al., 1996; Dib et al., 1996). However, all 21 markers located in the consensus deletion are mapped within a span of 3 cM (Fig.4). These results show that SEN16 is located within a 3-7 cM genetic interval at 16q24.3.

High resolution mapping of MX revertants of microcell hybrids of MCF.7 and SKBR-3 cells was hindered by the lack of polymorphism between recipient and donor alleles of most available markers. Of the markers listed in figure 4, only D16S498 and D16S413 were found to be polymorphic between donor and recipient chromosomes. Both of these markers were consistently deleted in all 5 revertant clones derived from senescent microcell hybrids of MCF.7 and SKBR-3 cells. D16S498 is part of the consensus deletion observed in rat revertant clones, while D16S413 is located just outside the consensus deletion (Fig.4). Thus the available data from microcell hybrids of human breast tumor cells are consistent with the mapping data in rat revertants and support the hypothesis that the same gene restores senescence in all four cell lines. To strengthen this conclusion, a derivative of chromosome 16q23-qter, carrying the mapped deletion in Rev 4, was introduced into MCF.7 cells. As expected, all 4 microcell hybrids obtained in this experiment were identical to the MCF.7 immortal parental tumor cells (data not shown). These results confirmed that the deletion mapped in Rev 4 abolishes the ability of 16q23-qter to restore

senescence in human tumor cells.

Discussion

We have mapped a cell senescence gene, SEN16, within a genetic interval of 3-7 cM at 16q24.3. The introduction and retention of a normal human chromosome 16, 16q22-qter or 16q23-qter into immortal mammary tumor cell lines restores cellular and colony morphology similar to normal breast epithelial cells and induces progressive retardation of proliferation leading to complete growth arrest. Recipient tumor cells carrying donor chromosome 16 are able to undergo 10-15 doublings before entering growth arrest. This residual growth potential allows sufficient expansion of chromosome transfer clones to identify viable senescent colonies, to extract DNA for analysis, to observe segregation of the donor chromosome in non-selective medium, and to isolate revertant clones in selective medium. Resumption of indefinite proliferation, concordant with the segregation of the donor chromosome, confirmed that restoration of senescence depends on retention of the donor chromosome. In control experiments, human chromosomes 7 or 13 had no effect on the proliferation of same cells.

In the course of these studies, we also developed an effective general strategy for the mapping of cell senescence/tumor suppressor genes. This strategy is based upon the identification of the smallest consensus deletion in the donor chromosome in independent revertant clones that arise in senescent microcell hybrids maintained in the selection medium. In the present study, the shortest shared consensus deletion that abolishes senescence gene activity is flanked by the markers, D16S486 and D16S413 which are separated by a genetic interval of less than 7cM at 16q24.3 (NCBI database). However, markers located in the consensus deletion are mapped within

a genetic interval of 3 cM at 16q24.3. A majority of the markers located in the consensus deletion are carried in two overlapping Yeast Artificial Chromosome (YAC) clones comprised of approximately 700kb of DNA (Our unpublished results). One of these YACs restores senescence when introduced into same tumor cell lines, confirming that SEN16 is located within the consensus deletion (manuscript in preparation).

The parallel use of rat and human cells as recipients was critical to the success of our mapping strategy. Heterospecific human/rat transfers made it easy to track human donor DNA markers, permitting high-resolution mapping which was not possible in human intraspecies hybrids, where only polymorphic markers can be used to map the donor chromosome. However, parallel low-resolution mapping in human cells and the transfer of a derivative of 16q23-qter from a rat revertant into human cells, served to confirm that the same gene is active in human as well as in rat recipient cells. Interestingly, SEN16 functions equally well in human and rat mammary tumor cells but does not restore senescence in mouse A9 cells, the host cell line of the mouse/human monochromosomal hybrid donor for chromosome 16.

Mapping of SEN16 was also facilitated by the identification and use of subchromosomal fragments of chromosome 16, which greatly reduced the number of markers required for deletion mapping. Both 16q22-qter and 16q23-qter, present in the respective mouse/human monochromosomal hybrid donor cell lines, are translocated onto mouse chromosomes, which are presumably transferred and retained along with the gpt tagged human fragments in the recipient tumor cell lines. Although the mouse carrier chromosomes have not been characterized, they are most likely different in the two subchromosomal hybrid cell lines which were generated independently. Thus, it is unlikely that cotransferred mouse DNA is responsible for the restoration

of senescence in the microcell hybrids reported here. In addition, the deletions mapped in the revertants strongly implicate the human fragment of the donor chromosome as responsible for the senescent phenotype. Moreover, whole cell hybrids between A9 cells and rat ovarian and brain tumor cells are invariably immortal (Kaur and Athwal, unpublished data) suggesting that no A9 chromosome can confer senescence in these cells. All senescent MCF.7 microcell hybrids entered growth arrest at approximately the same time, but post-replicative senescent MCF.7 cells carrying an intact normal chromosome 16 remained attached to plates longer than their counterparts carrying fragments (Table 1). The reason for this difference is not known but it could reflect a cell line specific effect of other co-transferred genes on chromosome 16.

Although this is the first report of a senescence gene on chromosome 16, the existence of tumor suppressor genes on this chromosome has been predicted from classical studies of loss of heterozygosity (LOH) on different tumors. In addition to breast carcinoma (Sato et al., 1991; Lindblom et al., 1993 and Tsuda et al., 1994), frequent deletions on 16q have been reported in prostate carcinoma (Latil et al., 1997), hepatocellular carcinoma (Tsuda et al., 1990) and Wilms tumors (Maw et al., 1992). The high incidence of allelic losses on 16q, in multiple tumors, suggest a universal role for gene(s) present on 16q in different cancers . Three regions on 16q, 16q22.2-16q22.1, 16q23.1-q23.3 and 16q24.3-qter, which show a high incidence of allelic imbalance, have been implicated in pathogenesis of breast cancer (Devilee et al., 1991; Cleton-Jansen et al., 1994; Tsuda et al., 1994; Lida et al., 1997). However, most frequent LOH has been observed at 16q24.3-qter irrespective of the stage of the disease (reviewed in Brenner and Aldej, 1997; Tsuda et al 1994; Devilee and Cornelisse 1994), suggesting that allelic imbalance at 16q24.3 may be an early event in the progression of breast cancer.

LOH analysis has been useful to identify loci that are deleted in tumor cells. However, it does not differentiate which loci are directly involved in tumor development and which may be lost coincidentally due to genomic instability associated with the malignant state. In contrast, functional complementation can distinguish whether a locus is involved in the suppression of tumorigenicity and/or metastasis in vivo or inhibition of cell growth and/or restoration of senescence in vitro (reviewed in Oshimura and Barrett 1997; Goyette et al., 1992) Our results show that a gene located at 16q24.3 is responsible for limiting the proliferative life span of breast tumor cells.

Several interesting genes, thought to be involved in the regulation of cell growth, have been mapped at 16q24.3. These include the renal dipeptidase gene, DPEP1 (Austruy et al., 1993), melanocortin stimulating hormone receptor gene, MCIR (Gantz et al., 1994), breast basic conserved gene, BBC1 (Adams et al., 1992), adhesion regulatory molecule, CMAR (Pullman and Bodmar, 1992), a metallopeptidase gene, PRISM1 (Scott et al., 1996), a gene named PISSLRE (Li et al., 1992) and the Fanconi Anaemia group A gene, FAA (Pronk et al., 1995). All these genes have been located within a 960kb DNA segment mapping between D16S3026 and D16S303 (Whitmore et al., 1998) which is distal to the region deleted in our immortal MX revertants clones. Thus, we have excluded all these genes as candidates for SEN16, based upon their map position or their retention in immortal revertants of senescent microcell hybrids.

The fact that SEN16 had essentially the same effect in four independently derived rat and human tumor cell lines suggests that it complements a common if not universal defect in mammary tumor cells. Since escape from senescence is considered to be involved in the conversion of non-malignant tumors to the malignant state, it is probable, that inactivation of SEN16 may be an essential step during the early stages of tumor progression required for unlimited clonal expansion

of the tumor. Direct testing for the role of SEN16 in the regulation of cell proliferation and mammary tumorigenesis will be possible once the gene is cloned.

Materials and Methods

Cell Lines and Growth Conditions:

Two human breast adenocarcinoma cell lines, SKBR-3 and MCF.7 (American Type Culture Collections, Rockville, MD), and two rat mammary tumor cell lines NMU (American Type Culture Collections), and LA7 (Ehmann et al., 1991) were used as recipients for microcell transfer experiments. Mouse/human monochromosomal hybrid cell lines RA7, RA13A and RA16A, RA16S3 and RA16S2, each carrying a *gpt* tagged normal human chromosome, were used as donors to transfer 7, 13, 16, 16q22-qter and 16q23-qter, respectively (R. S. Athwal, unpublished results). The human chromosomes in this panel of mouse/human monochromosomal hybrids came from normal diploid cell line GM03468A (Human Genetic Mutant Repository, Camden NJ).

All cell lines were routinely cultured in DF12 medium supplemented with 10-15% fetal bovine serum at 37^0 C in a 7.5% CO_2/ air atmosphere. The medium for selection and propagation of chromosome transfer clones and donor mouse/human monochromosomal hybrid cell lines contained 25 µg/ml mycophenolic acid and 70 µg/ml xanthine (MX medium).

Microcell Mediated Chromosome Transfer

Micronuclei formation in donor cells was induced by mitotic arrest with colcemid (0.2ug/ml) for 40 hours and microcells were prepared by zonal centrifugation as previously described (Athwal et al. 1985). Purified microcells were layered on top of a monolayer of recipient cells ($2x10^6$ / 10 cc dish) in the presence of phytohemagglutinin-P (PHA-P, 100µg/ml) and plates were incubated at 37^0 C for 15 min. The cell fusion was facilitated by the addition 1 ml of PEG 1500 (Boehringer Mannheim) using the standard procedure (Athwal et al., 1985). In each experiment microcells prepared from approximately $2x10^7$ cells were fused with $4\text{-}5x10^6$ recipient cells. After fusion, cells were cultured in non-selective medium for 24 hours, and then transferred to MX medium. Microcell hybrid colonies, observed during the ensuing period of 3-4 weeks, were either isolated individually or maintained in the same plates in MX medium.

Analysis of Chromosome Transfer Clones

Chromosome transfer colonies were maintained in MX medium and examined for cellular and colony morphology and photographed at regular intervals. The population doubling time in each colony was determined by counting cells, either under the microscope or in photomicrographs. The presence or absence of the donor chromosome in each clone and the location of deletions in the donor chromosome were determined by PCR analysis for donor chromosome specific DNA markers. All primer pairs for PCR amplification were either purchased from Research Genetics (Huntsville,AL) or synthesized commercially. Chromosome 16 specific markers used for mapping included: D16S303, 305, 402, 413, 422, 449, 476, 486, 488, 498, 520, 686, 2621, 2625, 2693, 2727, 2750, 2751, 2772, 2733, 2784, 2790, 2801, 2807, 2866,

3026, 3028, 3048, 3037, 3061, 3063, 3077, 3121, 3205 and 3318; WI-1435, 1728, 3181, 3217, 3661, 6143, 7624, 8422, 10279, 10391, 11335, 11775, 12410, 13072, 15502, 15838, 16080, 16844, 16952, 17119, 17574, 18220 and 18377; SHGC2485, 2489, 3238 and 11987; StSG 2389, 2700, 8033, 8665; SGC30619, 30711, 31012, 32044 , 33145, 33289 and 36958; KIAA0182 and 0233; TIGR-A00B17, A001Y24, A00Y26, A008S19, A00Q31 and A002Y45; D42053, D29571, D29107; U06088; X6563; EST00889; PRSM1 and BBC1.

Microcell hybrids of human cells were analyzed for polymorphic dinucleotide repeat markers (Dib et al., 1996) to distinguish among donor and recipient cell chromosomes. Briefly, the CA strand primers were end labeled with ^{32}P and used in PCR reactions as described (May and Weber, 1989). PCR products from chromosome transfer clones and parental cell lines were compared by separation in 6% polyacrylamide denaturing gels to distinguish donor and recipient alleles.

In microcell hybrids derived from rat recipient cells, human donor chromosome markers were detected by conventional PCR amplification. The PCR products were resolved on 2% agarose gels and visualized after staining with ethidium bromide. PCR amplifications were performed in a 25µl reaction volume containing PCR buffer, 200µM dNTPs, 2mM $MgCl_2$ and 10 picomoles primer pair, using the conditions as recommended by the primer supplier (Research Genetics, Huntsville AL).

The gpt gene present on the donor chromosome was detected by PCR amplification of a 700 bp segment using GPT1 and GPT2 primers as described (Kaur and Athwal, 1993).

Cytogenetic analysis

Metaphase spreads of microcell hybrids were prepared by standard methods and analyzed

by Fluorescent In Situ Hybridization (FISH). A biotinylated probe prepared from total human DNA was hybridized to metaphase spreads and was detected by staining with fluorescein labeled streptavidin (Oncor, Gaithersburg, MD) as recommended by the supplier.

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FIGURE LEGENDS

Figure 1: Photomicrographs of human and rat mammary tumor cell lines, showing cell morphology before and after the transfer of a normal human chromosome: **(A)** MCF.7, a human breast tumor cell line; **(B)** a senescent MCF/16 microcell hybrid containing donor chromosome 16 in MCF.7 cells; **(C)** a senescent MCF/16q23 microcell hybrid, containing donor chromosome 16q23-qter in MCF.7 cells; **(D)** an immortal MX revertant clone of microcell hybrid MCF/16; **(E)** LA7, rat mammary tumor cell line; **(F)** a senescent LA/16 microcell hybrid, containing donor chromosome 16 in LA7 cells; **(G)** a senescent LA/16q23 microcell hybrid, containing donor chromosome 16q23-qter in LA7 cells; **(H)** an immortal MX revertant clone of microcell hybrid LA/16q23.

Figure 2A : PCR Analysis of *gpt* and polymorphic dinucleotide repeat markers (D16S505, D16S515 and D16S498) in microcell hybrids of MCF.7 and SKBR-3 cells containing donor chromosome 16.

Lanes represent :

(1) RA16A, a mouse/human monochromosomal hybrid cell line containing chromosome 16; **(2)** MCF.7;

(3) A senescent MCF/16 microcell hybrid clone; **(4 & 5)** Immortal MX revertant clones of MCF/16 microcell hybrids; **(6)** SKBR-3; **(7)** A senescent SKBR/16 microcell hybrid clone; **(8-10)** Immortal revertant clones of SKBR/16 microcell hybrids.

2B: PCR amplimers of chromosome 16 specific markers (D16S2750, WI-15838, D16S2727 and D16S2733) in microcell hybrids of LA7 cells.

Lanes represent: (1) RA16S3, a donor hybrid containing chromosome 16q22-qter; **(2)** A

senescent LA/16q22 microcell hybrid clone; **(3)** Rev 2, an immortal revertant clone of an LA/16q22 microcell hybrid; **(4)** Rev 3, an immortal revertant clone of an LA/16q22 microcell hybrid; **(5)** RA16S2, a donor hybrid containing chromosome 16q23-qter; **(6)** A senescent LA/16q23 microcell hybrid clone; **(7)** Rev 4, an immortal revertant clone of an LA/16q23 microcell hybrid; **(8&9)** subclones of Rev 4 ; **(10)** LA7 recipient cells.

Figure 3: Metaphase spreads showing; **A)** donor hybrid RA16S2 containing human chromosomal fragment 16q23-qter; and **B)** a LA/16q23 microcell hybrid showing the presence of the introduced chromosome, 16q23-qter.

Figure 4: Physical and genetic map of human chromosome 16q23-qter, showing the location of the consensus deletion (shaded region) identified by analysis of DNA markers specific for chromosome 16 in immortal revertant clones (Rev 1, Rev 2, Rev 3 and Rev 4) of rat mammary tumor cells and in the donor mouse/human monochromosomal hybrid, RA16A. Markers found to be present (closed squares) or absent (open squares) are indicated in the respective lanes.

Table 1: Human chromosomes transferred and microcell hybrids recovered in different breast tumor cell lines.

Cell lines and donor chromosomes	*Number of colonies*			Length of survival*
	Total	*Senescent*	*Immortal*	
HUMAN				
MCF.7				
16	21	21	0	4 - 5
16q22-qter	5	5	0	2 - 3
16q23-qter	2	2	0	2 - 3
13	3	0	3	-
SKBR-3				
16	12	12	0	3 - 4
16q22-qter	5	5	0	3 - 4
16q23-qter	11	11	0	2 - 3
RAT				
LA7				
16	42	42	0	2 - 3
16q22-qter	35	35	0	2 - 3
16q23-qter	18	18	0	2 - 3
7	8	0	8	-
NMU				
16	2	2	0	2 - 3
16q22-qter	2	2	0	2 - 3
13	3	0	3	-

* Survival in months after colonies were observed or isolated

Fig.1

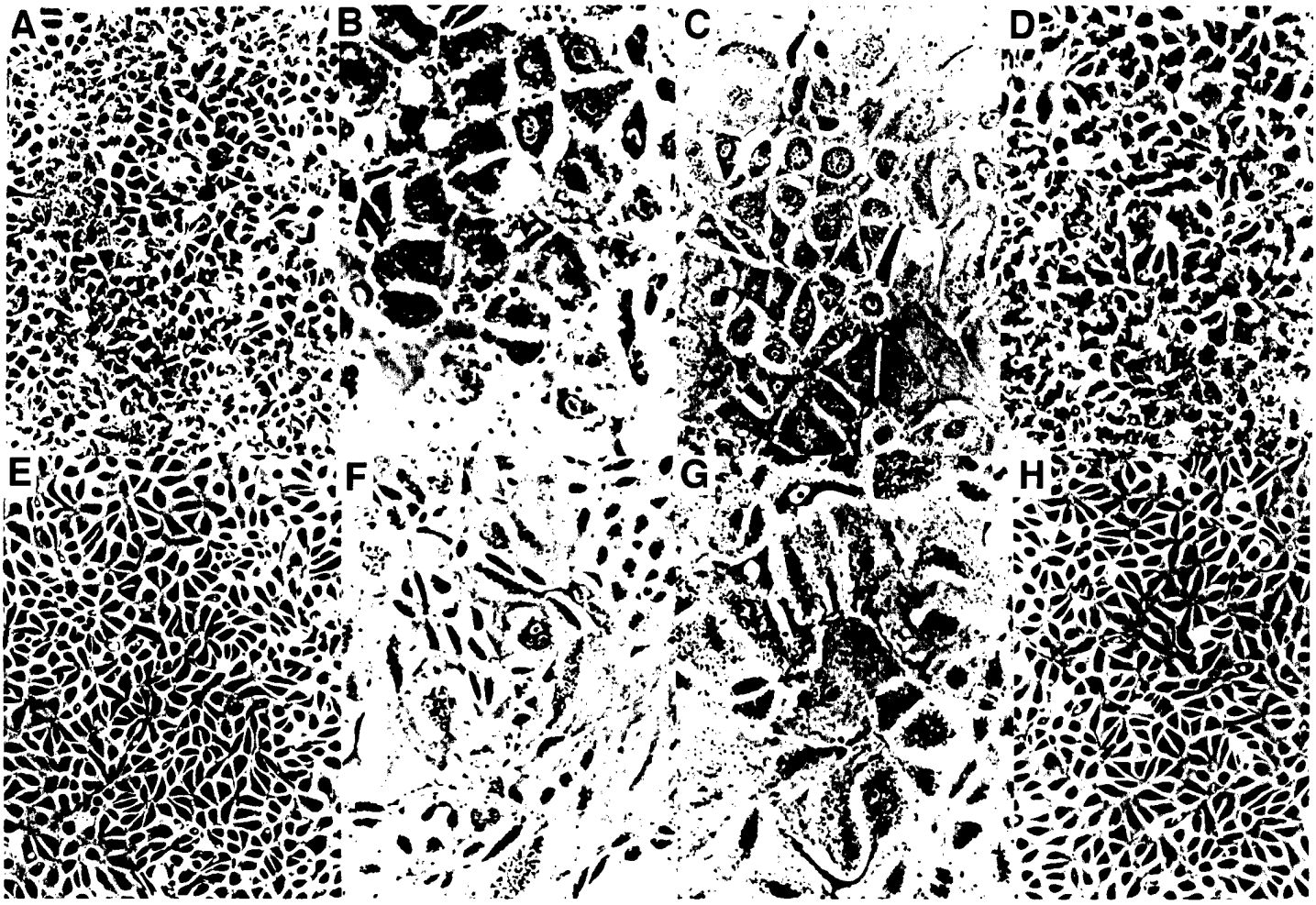

Figure 2

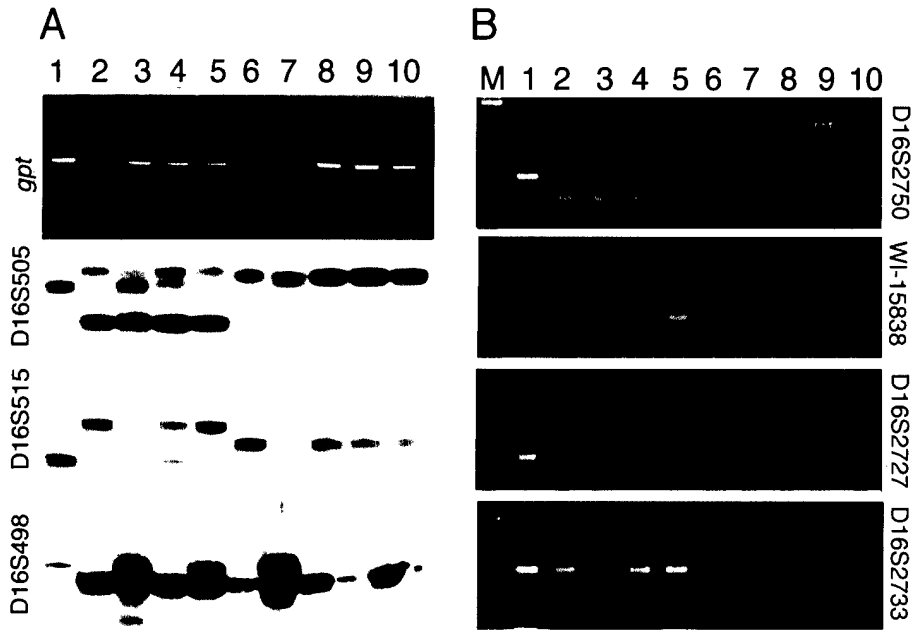
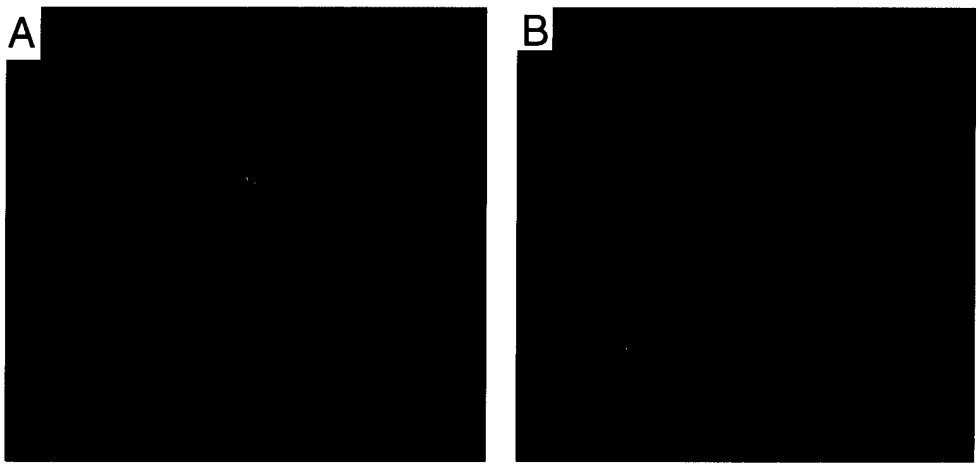
Fig 3