Cell Lines from grc Congenic Strains of Rats Having Different Susceptibilities to Chemical Carcinogens

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ABSTRACT

The growth and reproduction complex (grc) strains of rats have a 70-kilobase deletion in the major histocompatibility complex (MHC)-linked grc-G/C region that is associated with embryonic death, developmental defects, and an increased susceptibility to chemical carcinogens. To study further the effects associated with the deletion, fibroblastic cell lines from grc*, grc+, and grc~ rat embryos were developed: BIL-derived cell lines are congenic for the MHC and grc, whereas R16-derived cell lines are congenic for the grc alone. In early passages, all cell lines expressed the MHC class I antigen RTLA, had a diploid chromosome number, and did not display anchorage-independent growth or in vivo tumorigenicity. The grc~ cells [median population doubling time (PDT), 47 h] grew more slowly than the grc* (PDT, 30.5 h) and grc~ (PDT, 33 h) cells. All cells underwent crisis, but the crisis stage began earlier and lasted longer in the grc~ cells. The established grc* cell lines (PDT, 32.5 h) grew faster than the grc* (PDT, 48.5 h) and grc~ (PDT, 54 h) cell lines. Two of the three BIL-derived grc~ lines that survived crisis became anchorage independent in tissue culture and tumorigenic in histocompatible F1 rats (highly malignant fibrosarcomas) at passages 33 and 48, respectively; by contrast, none of the R16-derived grc~ cell lines transformed. None of 8 grc* or 8 grc~ cell lines that survived crisis displayed anchorage-independent growth or tumorigenicity under the same conditions up to passage 50. All of the established cell lines, including the two tumorigenic ones, expressed MHC class I antigens. Southern and Northern blot analyses of BIL-derived cell lines before and after crisis showed that they all constitutively expressed H-ras and Rb and that no cell line showed rearrangement, amplification, or overexpression of c-myc, H-ras, K-ras, Rb, and p53 either before or after crisis. These observations indicate that: (a) the homozygous grc~ deletion is necessary but not sufficient for in vitro transformation; (b) another genetic factor(s) required for transformation is linked to, or possibly in, the MHC; and (c) passage through crisis, spontaneous transformation, or carcinogen treatment does not alter the cellular expression of MHC class I antigens or of some oncogenes and tumor suppressor genes.

INTRODUCTION

Studies in humans and in animals have shown that neoplastic transformation is a multistep process (1–6) involving dominantly acting cellular oncogenes (7–10) and recessively acting tumor suppressor genes (1, 2, 8, 11, 12). The development of the neoplastic phenotype in cell culture systems is also a progressive multistage process (4) as shown by in vitro multistage chemical carcinogenesis studies (13–16) and by in vitro cell transfection studies using oncogenes or oncogenic viruses (5).

Rodent primary embryonic fibroblast cells are normal diploid cells that, after a variable number of divisions in vitro, enter a crisis phase where cell growth ceases or greatly declines. By serial passage in vitro, permanent cell lines can be established from the cells that survive crisis (17–20). The established cell lines have presumably developed a preneoplastic phenotype, and they can be further transformed by chemical, physical, or viral agents (5, 21). Immortalization and malignant transformation of primary rat embryonic fibroblast cells can also be achieved by transfection with one or more oncogenes, respectively (5).

The grc~ in the rat is a group of genes closely linked to the MHC (22, 23). Inbred and recombinant strains with deletions of approximately 70 kilobases in this region (grc~) (24, 25) have a variety of abnormalities: embryonic death (30% of homozygous offspring); developmental defects (decreased body size; fertility defects (male sterility and reduced female fertility); and increased susceptibility to chemical carcinogens (26–28). We propose that the grc~ strains have lost the genes that play an important role in maintaining normal cellular growth and development and that some of these genes may have the properties of tumor suppressor genes.

In the present study, two sets of embryonic fibroblast cell lines have been developed from strains congenic for the MHC + grc or for the grc alone in order to define their growth characteristics, to gain some insight into the genetic basis of transformation in vitro or in vivo, to study their responses to chemical carcinogens, and to examine their expression of MHC class I antigens and of some prototypic oncogenes and tumor suppressor genes.

MATERIALS AND METHODS

Animals. The rats were from our colony in the Department of Pathology, University of Pittsburgh (Table 1), and their derivation has been described in detail (28). The BIL/1 and BIL/2 strains differ at both the MHC and the grc, and all have the same non-MHC genes (from the BIL strain). The R16, R33, and R34 cell lines differ solely in the grc, or a part thereof, and all have the same non-MHC genes (from the ACP strain). All of the strains are monitored with allelic-specific monoclonal antibodies (29, 30).

Preparation and Characterization of Cultures. To obtain grc~ and grc~ cells, embryos, grc~ females were mated with heterozygous males. The grc~ embryos could be differentiated from their heterozygous littermates by measuring their body length (the grc~ embryos are smaller) and, thereafter, by phenotyping the MHC of the embryonic cell lines using flow cytometry. The grc~ embryos were obtained by mating grc~ animals. Embryos of 20-day gestation were obtained from anesthetized females under sterile conditions, decapitated, and vesiculated; the remaining carcass was minced finely with scalpels in Petri dishes. The tissue pieces were resuspended in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% NCS (GIBCO), 1-glutamine, vitamins, penicillin G, streptomycin, and amphotericin B (GIBCO) and seeded in T75 flasks (Corning Glass Co., Corning, NY). Examination of cell growth in different serum concentrations (data not shown) led to the selection of 10% NCS as optimal. The cells were incubated at 37°C in a humidified incubator with 10% CO2, and the medium was changed every 3 to 4 days. When they reached confluence, the cells were trypsinized (0.05% trypsin/0.53 mM EDTA, GIBCO) and passaged regularly at a reseeding density of 3 X 10^5 cells/T75 flask. Both the passage number and the number of population doublings (number of generations) were recorded. The cells were checked by mycoplasma agar plates (GIBCO), and no mycoplasma contamination was found. They were phenotyped with biotinylated, allele-specific monoclonal antibodies using the avidin-fluorescein isothiocyanate method and flow cytometry (Epic flow cytometer; Coulter Co., Hialeah, FL). The monoclonal antibody was directed against MHC class I antigens or of several oncogenes and tumor suppressor genes.

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2 To whom requests for reprints should be addressed.

The abbreviations used are: MHC, major histocompatibility complex; grc, the growth and reproduction complex; Rb, retinoblastoma; NCS, newborn calf serum; mAb, monoclonal antibody; PDT, population doubling time; DMSO, dimethyl sulfoxide; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; DMBa, 7,12-dimethylbenzanthracene; RFLP, restriction fragment length polymorphism.

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The relative survivals (survival as a percentage of solvent control) were plotted for cells exposed to MNNG for 24 h or exposed to DMBA for 72 h. The animals were followed for 3 months, and the times at which they reached confluence were noted. The cultured cells were centrifuged at 1000 rpm and resuspended in 2.5% glutaraldehyde. Following 1 h fixation, the tube was centrifuged, the fixative was removed, and the cells were resuspended in a 2% solution of Difco Bacto-agar in 0.1 M sodium cacodylate buffer at 100°C. The resuspended cells were immediately centrifuged and allowed to cool until the agar solidified. The plastic microfuge tube was cut with a razor blade; and the pellet was removed, placed in cacaoylate buffer, and processed as a tissue block and stained with uranyl acetate and lead citrate.

Molecular Analyses. In order to determine whether the expression of MHC genes and of various oncogenes and tumor suppressor genes was altered in any of the cell lines both before and after crisis, genomic DNA from the various cell lines was extracted by the procedure of Kunnath and Locker (35) and total RNA was isolated from cultured cells using RNAzol (Cinna/Biotech Laboratories, Houston, TX). The EcoRI-digested genomic DNA was analyzed as described previously (36). The total RNA (10 μg) was electrophoresed on a 1.5% agarose gel containing 6% formaldehyde and blotted onto a nitrocellulose membrane. The probes labeled by random priming using an oligolabeling kit (Pharmacia, Piscataway, NJ) were hybridized as described previously (36). They were: 5'pMK, a 230 nucleotide probe derived from a rat 77,-like gene (Fig. 1, A and C). The plating efficiencies were: grc- cells; 1.6%; grc+ cells, 3.2% (P > 0.2). The grc- cell lines grew faster than the grc+ lines, and was given the name of the strain from which it was derived (Table 1). This name is followed by specific descriptors: the serial number in parentheses to denote uniquely the line; and the passage number of that line denoted by p. If the cell line was treated with a carcinogen, the first letter of the carcinogen used comes after the passage number at which the line was treated, and this is followed, in turn, by a passage number indicating the number of passages of the cell line both before and after crisis, genomic DNA from the various cell lines was extracted by the procedure of Kunnath and Locker (35) and total RNA was isolated from cultured cells using RNAzol (Cinna/Biotech Laboratories, Houston, TX). The EcoRI-digested genomic DNA was analyzed as described previously (36). The total RNA (10 μg) was electrophoresed on a 1.5% agarose gel containing 6% formaldehyde and blotted onto a nitrocellulose membrane. 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Fig. 1. Phase-contrast microscopy of BIL rat embryonic fibroblasts (A-D). A, grc+ cell line n/n(16) before crisis showing normal fibroblastic morphology. B, the same cell line as in A, but after crisis; it maintains its fibroblastic morphology. C, grc~ cell line l/l(5) before crisis, showing normal fibroblastic morphology. D, the same cell line as in C but after crisis, showing transformed morphology. Toluidine blue staining of trypsinized cell lines (E, F) shows that the grc+ cells n/n(16)p31 are adhesive and in close contact (E), whereas the grc~ cells l/l(5)p45 are not (F); × 100.

more slowly (PDT, 47 h) than did the grc+ (PDT, 31 h) or grc+/- (PDT, 33 h) cell lines (P < 0.002); all cell lines had a modal chromosome number of 42; and none of the lines displayed anchorage-independent growth or in vivo tumorigenesis (Table 2). All cell lines expressed the MHC class I antigen RT1.A, but RT1.E was not expressed (Table 3).

Crisis Stage. Crisis was manifested in all cell lines by slowed growth, change in shape to very large and flat cells, and death of some cells. The grc~ cells, which were passed every 4–7 days at a split ratio of 1:2, entered crisis at median passage number 10.5 (Table 2). During crisis, these cells grew extremely slowly and took 2 or 3 weeks to reach confluence. After a 2–3-month period, they came out of the crisis stage at median passage number 20.5. The grc+ and grc+/- cells, which were growing faster and were passed twice a week at a split ratio of 1:3 or 1:4, entered crisis later than the grc~ cells: at median passage number 14.5 and 15.0, respectively (P < 0.001). The morphological changes during crisis were less severe than in the grc~ lines. During crisis, the cells were passed every 7–10 days, and both grc+ and grc+/- lines came out of crisis in 4–6 weeks at median passage number 21, which was the same as the grc~ cells (P > 0.6).

Cell Growth and Characterization after Crisis. The number of cell lines that survived crisis varied: 3/5 l/l; 3/5 n/n; 4/6 l/n; 3/3 R16; 2/2 R33; 3/3 R34; 1/1 R16(a/n); 3/3 R16/R34. The plating efficiencies were 13.5% for the grc~ lines and 7.4 and 7.8%, respectively, for the grc+ and grc+/- lines; they did not differ significantly from those before crisis (P > 0.03). The grc~ lines grew much faster (PDT, 32.5 h) than the grc+ (PDT, 48.5 h) and grc+/- (PDT, 54 h) lines (P < 0.002), which is the reverse of the situation before crisis, and showed variation in chromosome number (Table 2). In addition, the grc~ lines needed less serum than the grc+ and grc+/- lines: they grew optimally in 2.5% NCS, whereas the grc+ and grc+/- lines still required 10% NCS for optimal growth (data not shown). Two of the three l/l lines that survived crisis underwent spontaneous malignant transformation, as shown by loss of contact inhibition and rounded morphology, whereas none of the n/n or l/n lines transformed (Fig. 1, B and D). The n/n(16) cells grew in cohesive clusters, were polygonal, and had a small nucleus with a prominent nucleolus and a low nuclear:cytoplasmic ratio (Fig. 1E). By contrast, the l/l(5) cells grew as single cells without contact inhibition; they were polygonal, had a large nucleus with prominent nucleoli, were occasionally binucleate, and had a high nuclear:cytoplasmic ratio (Fig. 1F).

The n/n(16) cell line remained morphologically similar by electron microscopy before (p7) and after (p33) crisis (Fig. 2, A and B). The cytoplasm was abundant and had much dilated rough endoplasmic reticulum. The cells grew in close contact, but no intercellular junctions were seen. The l/l(4) cell line was examined before (p30) and after (p54) transformation. Before transformation, the cells were similar to the n/n strain (Fig. 2C). After transformation, the cells developed morphologically malignant characteristics: a large irregular nucleus; prominent nucleoli; a high nuclear:cytoplasmic ratio; and numerous cilia on the cell membrane (Fig. 2D).

All the established cell lines were tested for anchorage-independent growth and in vivo tumorigenesis up to passage number 50. Anchorage-independent growth and in vivo tumorigenesis developed in two of the three l/l cell lines (l/l(5) at passage 34 and l/l(4) at passage 48) but not in the three n/n or the four l/n cell lines (Table 2). The cloning
Table 2. Cell lines established and their growth characteristics

<table>
<thead>
<tr>
<th>RT1 specificities</th>
<th>Population doubling time (h)</th>
<th>Passage no. (generation no.)</th>
<th>Modal chromosome no.</th>
<th>Anchorage-independent growth</th>
<th>In vivo tumorigenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>Before crisis &lt;br&gt;(p5-9)</td>
<td>After crisis &lt;br&gt;(p26-32)</td>
<td>Beginning of crisis</td>
<td>End of crisis &lt;br&gt;(p5-9)</td>
<td>No. (passage) % in mode</td>
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</table>

* By phase contrast microscopy, the cells were all fibroblasts.
*p, passage number.
+ Sixty % of BIL-derived cell lines and 100% of R16-derived cell lines survived crisis.
* Fibrosarcoma. Each of the three injection sites measured approximately 50 x 30 x 30 mm at days 18-21 after injection into BIL rats. Maximal growth of 1/1 (4) occurred in 42 days and that of 1/1 (5), in 20 days.
* Heterozygous male used to propagate the R16 strain (see "Materials and Methods").

Table 3. Expression of MHC class I antigen RT1.A before and after crisis

<table>
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<th>Cell line</th>
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<th>After crisis</th>
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<td>R16/a (25)</td>
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* Two l/ , two n/n, and two ln cell lines did not survive the crisis phase.
* mAb 70 (anti-E+) did not react with any RT1.E+ cell lines.

efficiency in soft agar was 10% for the l/ (4) line and 20% for the l/ (5) line. The tumors in the histocompatible BIL strain following injection with the l/ (4) or l/ (5) cell lines grew very rapidly to approximately 50 x 30 x 30 mm: in 20 days for l/ (5) and in 42 days for l/ (4). They were extremely aggressive fibrosarcomas that consisted mainly of spindle cells with irregular nuclei and prominent nucleoli. The mitotic activity was high ( > 5 mitoses/high power field), but no metastases were found. Collagen was identified using the trichrome and reticulin stains, and intracellular glycogen was identified by the PAS stain. Immunohistochemical staining for desmin was negative, ruling out a smooth muscle origin for the tumor.

None of the R16-derived cell lines developed anchorage-independent growth or in vivo tumorigenesis. All three types of established cell lines, including the two tumorigenic lines, expressed the MHC class I antigen RT1.A but not RT1.E (Table 3). Cell lines derived from tumor explants also strongly expressed RT1.A (92% positive for l/ (4)-derived tumor cell line and 86% positive for l/ (5)-derived tumor cell line). No class II antigens were expressed by these fibroblastic cell lines (data not shown).

Treatment with Chemical Carcinogens. Treatment of both early-passage (p5-7) and established (p30, p31) cell lines with MNNG or with DMBA resulted in an increasing toxicity from 0.1 to 32 µg/ml. The survival curves of the grc-, grc+, and grc+/~ cells were similar, with DMBA resulted in an increasing toxicity from 0.1 to 32 µg/ml.

The survival curves of the grc~, grc+, and grc+/~ cells were similar, with DMBA resulted in an increasing toxicity from 0.1 to 32 µg/ml.

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Fig. 2. Electron micrographs of cell lines examined before and after crisis. A, grc+ line n/n(16)p7 before crisis. The nuclei are small, the cytoplasm contains relatively few organelles, and very few villi project from the cell surface. B. The grc+ line n/n(16)p33 after crisis. The nuclei are large, irregular, and have prominent nucleoli; and the cytoplasm has abundant organelles. C, grc+ line l/l(4)-p30 after crisis but before transformation. Their morphology is similar to the n/n(16) cell line in B. D, grc- line l/l(4)p54 after transformation. The nuclei are more irregular, there are many long slender microvilli, and the cells lose their cohesion. X1680.

Table 4 Expression of MHC class I antigen RT1.A on established cell lines before and after carcinogen treatment

<table>
<thead>
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<th>Cell line</th>
<th>RT1.A</th>
<th>mAb</th>
<th>Untreated</th>
<th>Solvent</th>
<th>MNNG treated (µg/ml)</th>
<th>DMBA treated (µg/ml)</th>
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<td>43</td>
<td>62</td>
<td>74</td>
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Table 4 Cell line died.

Molecular Analyses. Southern and Northern blotting was done to determine whether there was any alteration in the cellular expression of MHC class I antigens or of several oncogenes and tumor suppressor genes following crisis or following transformation. Such alterations in the expression or the structure of MHC class I genes (38-40), c-myc and ras oncogenes (41-45), and Rb and p53 tumor suppressor genes (46-50) have been observed in different types of malignancies. The EcoRI-digested DNA and total RNA from the two tumorigenic grc- cell lines l/l(4) and l/l(5) were analyzed before crisis (passages 5-7), after crisis (around passage 30), high passage number (around passage 50), after treatment with the carcinogens MNNG or DMBA, and after derivation from tumor explants. The grc+ cell line n/n(16) and the grc+/~ cell line l/n(7) served as controls.

The mouse general class I probe pAG64c detected strong MHC class I transcripts and numerous RFLP bands in both the l/l and n/n cell lines and showed that each cell line had a distinct RFLP pattern. There was, however, no discernible change in either the transcripts or the RFLP patterns of the cell lines at different stages of growth. The 5'pMK probe, which was derived from a TL-like gene within the grc region (36), hybridized to a 5.8-kilobase band in n/n and l/n, but not in l/l cells, as expected; Northern analysis using this probe did not detect any transcripts. A rat tumor necrosis factor α probe hybridized
to a 3.7-kilobase DNA band in all cell lines examined, but it did not detect any RNA transcripts.

Southern analysis with the c-myc probe detected a 17-kilobase band; with the H-ras probe, 14- and 23-kilobase bands; and with the K-ras probe, 2-, 4.7-, 9.1-, and 10-kilobase bands. The RFLP patterns were identical in all cell lines tested. Only an H-ras transcript was detected by Northern analysis. There was no indication of amplification, rearrangement, or overexpression of these genes when the two l/l cell lines were compared with the n/n and l/n cell lines. There was no rearrangement or deletion in the Rb or p53 loci: the p53 probe detected 1.7-, 3.6-, 3.8-, and 10-kilobase bands and the Rb probe detected 3.8- and 15-kilobase bands in every cell line examined. All cell lines constitutively expressed a small amount of the Rb gene mRNA, but no p53 transcript was detectable.

**DISCUSSION**

The MHC-linked deletion in the grc~ strains is a necessary condition for spontaneous in vitro transformation of cell lines, but whether it is sufficient in itself probably depends upon either the size of the deletion or an additional gene in the MHC region. Resistance to chemical carcinogens (the rcc~ locus) in vivo segregates with the grc in two unique sets of recombinant congenic strains (28) that have different non-MHC genes; hence, spontaneous transformation of the cell lines derived from these animals is a function of the grc or MHC-grc region only. In addition, both susceptibility to carcinogens in vivo and transformation of the derived cell lines in vitro are recessive traits, so the crucial gene affecting transformation may be a tumor suppressor gene. The inability of the carcinogens MNNG and DMBA to induce transformation in the grc~ and grc+/- cell lines further supports the hypothesis that the loss of a gene in the deletion rather than the activation of an oncogene is the critical factor in transformation.

The BIL-derived cell lines carrying the grc~ deletion, which transform spontaneously, are congenic for both the MHC and the grc, whereas the R16-derived grc~ cell lines, which do not transform, are congenic for the grc only. In addition, 60% of the BIL-derived cell lines survive crisis, whereas 100% of the R16-derived cell lines survive. The deletion in the BIL-derived strains is approximately 70 kilobases (25), but its size in the R16-derived strains is not known. Thus, there could be an additional gene that is lost in the BIL-derived grc~ cell line but is not lost in the R16-derived grc~ cell line. Alternatively, there could be a gene in the MHC of the BIL-derived cell line that acts as a positive stimulus to malignant transformation, i.e., as an oncogene, that is absent in the R16-derived cell line. We favor the former hypothesis, since some evidence from in vivo susceptibility to chemical carcinogens suggests that two closely linked genes may be involved in the rcc~ locus (28). The susceptibility of F1 hybrids between strains susceptible and resistant to chemical carcinogens is 25–40% of the susceptibility of the grc~ homozygotes. This observation suggests that at least two genes may be involved in the rcc locus and that different deletions are present that complement each other in the F1 hybrid leading to an increase in susceptibility over that expected from the action of a single recessive gene.

There was no significant change in MHC class I antigen expression on any of the cell lines, both grc~ and grc+, before or after crisis, after spontaneous transformation, or after carcinogen treatment in vivo. This observation suggests that such a change does not reflect the growth potential of the cell, including its tumorigenicity. The literature in this field is mixed. Some studies show that many rodent and human tumors have a marked reduction in expression of MHC class I antigens that is correlated with reduced immunogenicity (38–40, 51, 52). Concomitantly, in some systems reexpression of MHC class I antigens following transfection with a class I gene led to rejection or reduced growth of the tumor (53–57). By contrast, a variety of other reports (58–60) does not confirm this correlation, and our experimental results concur with this lack of correlation. In addition, in our experimental system immortalization, spontaneous transformation, and carcinogen treatment did not alter the expression or the structure of several prototypic oncogenes and tumor suppressor genes. Thus, the process of transformation and tumorigenicity is a subtle and complex one, and there does not appear to be any clear correlation between specific cellular changes and the activity of a specific gene.

The grc~ cell lines before crisis, both BIL and R16-derived, grow more slowly than the grc+ and grc+/~ cell lines, and this pattern occurs in the intact animal as well. Thus, the loss of the dw-3+ gene, which affects body size, along with the rcc+ gene, which affects resistance to chemical carcinogens, in the deletion that occurs in the grc~ strains manifests itself in the same way both in vivo and in vitro. It also suggests that a second gene that may affect in vitro transformation would map to the right of dw-3+ (28) and would be lost in the BIL deletion but not in the R16 deletion. The nature of these genes is not known, but some of them may affect the receptors for various growth hormones. Endocrinological (22, 61) studies show some abnormalities in grc~ rats and electron microscopic (62–64) studies show membrane defects in grc~ rats.

In summary, MHC-linked genes play an important role in both normal and abnormal growth. The segment of the chromosome carrying the MHC and its linked region has been highly conserved throughout evolution, and it controls similar phenomena in a variety of species (65). Its role in the control of growth may lie in the presence of tumor suppressor genes and of genes that affect a unique step in the transformation process (and possibly also suppressor-type genes), the loss of which is a critical event in the induction of the neoplastic process.

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**REFERENCES**


Cell Lines from *grc* Congenic Strains of Rats Having Different Susceptibilities to Chemical Carcinogens

Di Lu, Heinz W. Kunz, Mona F. Melhem, et al.


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