Tumor Suppressor Genes: The Puzzle and the Promise

RUTH SAGER

Tumor suppressor genes are wild-type alleles of genes that play regulatory roles in cell proliferation, differentiation, and other cellular and systemic processes. It is their loss or inactivation that is oncogenic. The first evidence of tumor suppressor genes appeared in the early 1970s, but only within the past few years has a wealth of new information illuminated the central importance of these genes. Two or more different suppressor genes may be inactivated in the same tumors, and the same suppressors may be inactive in different tumor types (for example, lung, breast, and colon). The suppressor genes already identified are involved in cell cycle control, signal transduction, angiogenesis, and development, indicating that they contribute to a broad array of normal and tumor-related functions. It is proposed that tumor suppressor genes provide a vast untapped resource for anticancer therapy.

ASPECTS OF THE TUMOR SUPPRESSOR GENE LITERATURE have been discussed by several investigators within the past few years (*1–8*), but new findings have clarified and dramatized the importance of these genes in the inhibition of tumor growth. With improved detection methods, the loss or inactivation of putative tumor suppressor genes is being found in every type of solid tumor. In particular, the new findings provide some answers to the long-standing question: how does the loss of gene function promote cancer?

Cancer results from mutations that disrupt the harmonious checks and balances that regulate normal cellular growth and development. These mutations arise in two classes of interacting genes: those that facilitate cell growth and tumor formation, in which mutation or overexpression is oncogenic, and those that inhibit these processes (the tumor suppressor genes) whose loss is oncogenic.

The interaction of positive and negative signals to maintain homeostasis is a motif seen everywhere in biology. In cancer, where homeostasis is impaired, mutations of both tumor promotor and tumor suppressor genes are part of the malignant process. Tumor suppressor genes block the oncogenic events that drive malignant progression.

Genetic mechanisms of tumor suppression operate within the cell and in systemic interactions between cells of different types. Within the cell, certain properties are at risk in the neoplastic transformation, clearly seen when one compares normal and tumor cells. Among them are chromosome stability, the capacity to undergo terminal differentiation, and the control of proliferation. There is evidence to be discussed below that tumor suppressor genes are active in these areas.

Systemic interactions include (i) communication between cells through junctional connections, steroid hormones, or secreted signal peptides (for example, growth factors or cytokines); (ii) immune surveillance; (iii) regulation of angiogenesis, that is, blood supply to tumors; and (iv) regulation of tumor invasion, including changes in expression of matrix components, proteases, and antiproteases. Genetic changes underlie most if not all of these altered systemic interactions, but the effects may be indirect. Altered responses to secreted signal peptides, for example, may result from mutations affecting receptor structure; improved tumor invasiveness may result from mutations in genes encoding specific proteases or antiproteases. The systemic aspects will not be included here for lack of space, but they merit full discussion to provide a balanced overview of the subject.

Detection of Tumor Suppressor Genes

Oncogenes are identified by their positive role in the transformation of appropriate host cells (*9*). Tumor suppressors, on the other hand, have an essentially negative effect, blocking transformation and driving cells toward normality. The difficulty in devising strong selection procedures for this negative phenotype has been one of the principal deterrents in tumor suppressor research.

Until recently, all of the evidence supporting the existence of tumor suppressor genes was indirect. Even today only a few such genes have been cloned and sequenced, but the multiple lines of supporting evidence have become very persuasive.

Tumor formation is suppressed in N × T cell hybrids. Tumor suppression was first demonstrated in murine cell hybrids produced by fusions between normal and tumor cells (N × T hybrids) (*1–7*). The tumor cells used were established from a carcinoma, a polyoma virus–induced sarcoma, a chemically induced sarcoma, and a lymphoma; the normal cells were L cells or primary fibroblasts. With most combinations, it was evident that the N × T hybrids were initially nontumorigenic, like the normal parent; subsequently, as the hybrid clones were propagated in culture, chromosomes were lost, and reversion to tumor-forming ability occurred.

Because reversion occurred, the genetic basis of tumor suppression remained unclear for several years. Subsequently, it was demonstrated unambiguously that chromosomes carrying tumor suppressor genes were lost when suppressed hybrids regained tumor-forming ability.

Hybridomas would seem to be an exception, since these cell hybrids, from fusions of myeloma cells with normal B lymphocytes, are still malignant. However, the intense selection that precedes

The author is Chief of the Division of Cancer Genetics at the Dana-Farber Cancer Institute, and Professor of Cellular Genetics in the Department of Microbiology and Molecular Genetics at Harvard Medical School, Boston, MA 02115.

establishment of hybridoma clones suggests that they represent rare variants (*7*).

Cell fusion is still a powerful tool for certain purposes, even in this era of molecular technologies. In general,

1) N × T hybrids can be used to identify the chromosome carrying an effective tumor suppressor gene. For example, in N × T hybrids from fusions between tumorigenic BHK cells and normal human fibroblasts, tumor suppression caused by inhibition of angiogenesis was correlated with the presence of human chromosome 1 (*10*).

2) N × T hybrids can be used to study expression of any markers by which the parents differ. For example, hybrids from fusions between normal and tumor-derived human mammary epithelial cells have been used to demonstrate expression of various genes of normal cell origin, as well as suppression of tumor formation (*11*).

The cell fusion method has been updated by microcell transfer, in which one or a few chromosomes within a reconstituted membrane from normal cells are transferred by membrane fusion to recipient tumor cells (*12*). When tumor suppression occurs, this method permits cytogenetic identification of individual donor chromosomes carrying tumor suppressor genes and reduces the complexity of gene interactions about 20-fold.

Experiments in which microcell transfer has been used to identify human chromosomes carrying putative suppressor genes are listed in Table 1. Most studies to date have focused on chromosome 11 because evidence from N × T hybrids had identified chromosome 11 as an effective suppressor of HeLa cell tumorigenicity in nude mice (*13*) and because deletions in 11p had been identified cytogenetically in Wilms' tumors (*14*). Chromosome 1 has also been recognized as a carrier of tumor suppressor genes by cell hybrid analysis (*10*), and 3p carries a deletion seen cytogenetically in small cell lung cancer (*15*), in renal cell carcinomas (*16*), and in cervical carcinomas (*16*).

Suppressor genes can be identified in heritable tumors. Since the early 1970s, Knudson (*2*) and others have stressed the usefulness of heritable tumors to investigators searching for cancer-related genes. In 1977, Mulvihill (*17*) presented a list of about 200 genes, recognized in pedigrees as autosomal dominant or recessive. The paradigm of success with this approach is the retinoblastoma gene, *RB*. Unexpectedly, however, the *RB* mutation, dominant in pedigree analysis, turned out to be recessive at the cellular level (discussed below). The loss of function is oncogenic, and it is this novel discovery that has revolutionized current thinking about cancer genes.

Other loss-of-function genes also appear as dominant in pedigrees and are recessive at the cellular level. Heterozygous progeny inherit a cancer-related mutant gene from one parent and the wild-type (normal) allele from the other parent. For the mutant phenotype to be expressed, the normal allele must be lost, and this occurs sporadically during cell proliferation. These two mutations, or losses, comprise the "two-event" paradigm proposed by Knudson (*2*). Dominance in pedigrees depends on a very high probability that the normal allele will be lost or inactivated in a potential tumor cell. This probability depends on a number of independent variables, including the size of the gene, the pool size of expressing cells, and the selective advantage that permits clonal growth of the tumor.

Table 1. Tumor suppression by chromosome transfer. NR, not reported.

Tumor cell recipient	Chromosome transferred		Reference
	Suppressive	Nonsuppressive	
HeLa (cervical)	11	X	(*66*)
Wilms' (renal)	11	X, 13	(*67*)
SiHa (cervical)	11	NR	(*68*)
A204 (rhabdomyosarcoma)	11	NR	(*68*)
HHUA (endometrial)	1,6,9	11	(*68*)
YCR-1 (renal)	3p	11	(*68*)
SK-N-MY (neuroblastoma)	1	11	(*68*)

Without describing the course of retinoblastoma research, since numerous reviews exist (*2, 18*), I will stress a few points that are especially relevant to detection of tumor suppressor genes. (i) The *RB* gene was initially mapped by cytogenetic means, recognition of deletions in 13q. (ii) The discovery of tight linkage to the esterase D gene facilitated localization in 13q14, and the subsequent cloning of esterase D provided the first material for chromosome walking to clone *RB*. (iii) Restriction fragment length polymorphism (RFLP) analysis was used to demonstrate the transition from heterozygosity to homozygosity of the *RB* gene in comparisons of DNA from normal and tumor tissues and to assess the chromosomal mechanisms responsible for this transition. The successful use of RFLP analysis in detecting loss of heterozygosity with retinoblastoma specimens led to widespread use of this method with other cancers (discussed below).

In retinoblastoma, loss of the *RB* gene is the only consistent genetic change that has been associated with the tumor, and therefore the two-event paradigm appears to fit very well. Whether the loss of *RB* is sufficient to produce retinal tumors without other accompanying mutations remains unanswered as yet.

Much of the research on *RB* before its cloning was paralleled by studies of Wilms' tumor, an embryonic kidney tumor (*14, 19*). A deleted region on chromosome 11p13 was associated with the WAGR syndrome of aniridia, genitourinary anomalies, mental retardation, and high risk of Wilms' tumor, by cytogenetics (*14*) and RFLP analysis (*20*). In addition, the Beckwith-Wiedemann syndrome, another developmental anomaly that is associated with a high risk of Wilms' tumor, has been mapped to 11p15 (*21*). Thus, the data implicate two Wilms' tumor suppressor genes, one at 11p13 and one at 11p15, but whether they act independently is not known. Currently, an added problem in mapping Wilms' tumor has arisen, as a result of evidence that in some families, the occurrence of Wilms' tumor does not map to either 11p13 or 11p15 (*22*). Thus, a novel Wilms' tumor predisposition gene, not yet mapped, has been identified by pedigree analysis. Its relation to the loci on chromosome 11 remains unknown.

In its simplest form, the two-hit paradigm may be unique to retinoblastoma, in which only a single gene has been associated with tumor suppression. If the two-hit analysis is restricted to individual genes, then the paradigm is useful in identifying other tumor suppressor genes; both wild-type copies must be lost for suppression to occur. The identification of tumor suppressor genes by the popular loss of heterozygosity (LOH) test, discussed below, is applicable in this context. The surprise in the recent Wilms' tumor mapping data lies in the evidence that more than one suppressor gene may be involved in this pediatric tumor. Analogous observations have been made with other tumors. For example, in multiple endocrine neoplasia type 2A (MEN2A), familial predisposition has been linked to chromosome 10 (*23*), but loss of heterozygosity has been found on chromosome 1p (*24*). Suppression of angiogenesis has been associated with loss of chromosome 1q in somatic cell hybrids (*10*), but also with expression of thrombospondin, encoded by a gene on chromosome 15 (*25*).

Adult cancer is a multigene process, in which tumor promotor genes as well as suppressors play essential roles. Individual suppressor genes may follow the two-event paradigm, and therefore the LOH, as discussed below, can aid in identifying and mapping new tumor suppressor genes. But the two-event paradigm is not appropriate as a full description of the genetic changes in adult tumors.

Even in pediatric tumors, the new complications in mapping of Wilms' tumor suggest multigene involvement in suppression.

In studies of nonhereditary (sporadic) colorectal tumors (*26*), DNA was extracted from thick sections of frozen specimens in which the tumor cell content was found to be high (judged by microscopic examination of alternate thin sections). Five distinct genetic changes have been identified so far, and with some exceptions, they occur sequentially, supporting the cumulative, multistep nature of tumor progression. An early change, also seen in the inherited disease familial adenomatous polyposis or FAP (*27*), is a deletion in chromosome 5q. In FAP, the multiple polyps that form are benign, but there is a strong predisposition to progression of some polyps to become carcinomas. Demethylation at cytosine residues and activating mutations in K-*ras* were found in benign adenomas, and in the transition to malignant tumors there were losses of chromosomes 17p (17p13) and 18q. Since deletions would be expected to include genes with tumor suppressor activity, at least three such genes are implicated in colorectal cancer. One of them at 17p13 is probably the p53 gene (*26*), but the others have not yet been identified.

In lung cancer, where primary tumors are often small and heterogeneous, the method of choice for characterizing changes at the DNA and RNA levels has relied on the establishment of cell lines from tumor cell populations obtained after surgery (*15*). Characteristic genetic changes in lung tumors include deletions in three regions: chromosome 3p12–22, 13q14, and 17p13, as well as overexpression of one of the *myc* genes (c-*myc*, N-*myc*, or L-*myc*), and activation of other proto-oncogenes such as c-K-*ras* and c-*raf* (*15*).

The tumor suppressor data are particularly impressive because three independent loci are often simultaneously deleted or mutated in these lung tumors. Chromosome 3p changes have been found in most small cell lung tumors and in over 50% of non–small cell lung tumors. Abnormalities involving 13q14 (*RB*) have been found in 70% of small cell lung cancers and 60% of non–small cell lung cancers; and in 17p a conservative estimate of tumors carrying mutant or deleted p53 is about 60% (*15*).

In familial malignant melanoma and its precursor, the appearance of dysplastic nevus, a gene (*CMM*) located by pedigree analysis to chromosome 1p36 is deleted as a late event in melanoma progression (*28*). Other genes must have mutated earlier, and karyotypic abnormalities of chromosomes 1, 6, 7, and 9 have been reported (*28*). Thus, melanoma resembles colorectal and lung cancer in containing several mutant cancer-related genes including putative tumor suppressors.

Identification of new tumor suppressor genes may be anticipated from other pedigree studies as well, such as tumors of neuroectodermal origin (*2*). Inherited predisposition to these tumors focuses attention on a new arena for molecular genetic investigation.

Allelic loss (LOH) can be detected by RFLP analysis. The development of RFLP analysis, originally conceived as a method to use polymorphisms in DNA as linkage markers (*29*), has been applied to the search for chromosomal losses that lead to allelic homozygosity or hemizygosity (*30*). There are already some 1800 known RFLP markers, covering all the human chromosomes at an average spacing of about 10 million bp, which is roughly equivalent to one RFLP every 100 genes.

RFLP analysis can be applied to establishing whether an allelic loss is associated with a particular clinical syndrome. To determine whether the LOH involves an inherited disease, investigators use pedigree analysis to examine DNA from at least three generations of families that are polymorphic for the markers available in the chromosomal region of choice. In looking for somatic changes occurring in tumor development in order to detect homozygosity, it is sufficient to compare DNAs from normal and tumor-derived cells of the same patient.

Nonetheless, pedigree analysis in LOH experiments can establish whether the lost allele was from the unaffected parent, as assumed, or from the parent transmitting the predisposition. Also, in the absence of pedigree analysis, there is no evidence whether a germinal mutation was involved.

A priori one cannot predict whether a tumor suppressor gene may be identified more readily by linkage analysis, by LOH mapping, or by other methods. Nonetheless, it should be stressed that rare hereditary predispositions can bring to light additional cancer-related genes not previously recognized (*31*). To judge from the record to date, most if not all of them will be tumor suppressors.

Analysis with LOH has been applied to many different tumor DNAs, for which it provides initial mapping of putative tumor suppressor genes, but additional methods are required for precise localization and specific identification. Also, the occurrence of LOH provides no evidence about the tumor phenotype or the mechanism of LOH. The method demonstrates the loss of a chromosomal region, but the mechanism may not be structural deletion. For example, in one retinoblastoma study, two-thirds of tumors showed nondisjunction or mitotic recombination rather than structural deletion (*18*).

Nonetheless, the importance of structural rearrangements is obvious when one observes the typically complex karyotypes of tumor cells. Experimental studies demonstrating the frequency of large deletions in tumor cell lines also support the likelihood that structural deletions play a prominent role in gene inactivations occurring during tumor growth (*32*).

Table 2 lists some tumor types for which LOH data have been described. Virtually all of them correspond to tumors that were on Mulvihill's 1977 list of hereditary tumors (*17*). However, the data in Table 2 are based largely on RFLP analysis of normal and tumor DNA, not upon pedigrees. The parallel provides an unexpected confirmation of a proposal stressed by Knudson (*2*) that the same mutant gene may be involved either in a hereditary predisposition in which one allele has been mutated prezygotically, or in a sporadic tumor, in which mutations in both alleles occurred postzygotically in somatic cells.

The new findings summarized in Table 2 illustrate the need to reorient our thinking about cancer. (i) Several different cancer-

Table 2. Loss of heterozygosity (LOH) in human tumors.

Chromosome	Type of cancer
1p	Melanoma (*69*); MEN2 (*23*); neuroblastoma (*23*); medullary thyroid carcinoma (*23*); pheochromocytoma (*23*); ductal breast carcinoma (*70*)
1q	Breast carcinoma (*71*)
3p	Small cell lung cancer (*15*); renal cell carcinoma (*16*); cervical carcinoma (*16*); von Hippel-Lindau disease (*31*)
5q	Familial adenomatous polyposis (*27*); sporadic colorectal cancer (*26*)
11p	Wilms' tumor (*14*); rhabdomyosarcoma (*23*); breast carcinoma (*72*); hepatoblastoma (*23*); transitional cell bladder carcinoma (*23*)
11q	MEN-1 (*23*)
13q	Retinoblastoma (*2*); osteosarcoma (*73*); small cell lung cancer (*15*); ductal breast cancer (*74*); stomach cancer (*23*)
17p	Small cell lung cancer (*15*); colorectal carcinoma (*26*); breast cancer (*75*); osteosarcoma (*73*)
18q	Colorectal carcinoma (*26*)
22	Meningioma (*23*); acoustic neuroma (*23*); pheochromocytoma (*23*)

related genes may be mutated or lost in the same tumor; (ii) the same genes may be mutated or lost in different kinds of tumors; and (iii) more than one tumor suppressor gene may be lost during the progression of individual tumors. It will take some time and more knowledge before the full significance of these results can be assimilated. It is already clear, however, that cancer is a genomic disease characterized by an accumulation of mutations and rearrangements in cells undergoing continuous selection. Thus tumor progression is a highly accelerated evolutionary process that occurs within a single lifetime (*33*).

Tumor suppressor genes can be identified by molecular cloning. Despite the lack of strong selection systems, progress has been made in cloning some tumor suppressor genes. Genes affecting nuclear functions (*RB* and p53) as well as signal transduction pathways in the cytoplasm (K*rev-1*) have been cloned.

The retinoblastoma gene has been cloned not only at the cDNA (*34*) but now at the genomic (*35*) level, a giant task because of the size of the gene (200 kb), the complexity of its organization, and the absence of a biological assay. Twenty-seven exons have been identified, accounting for the total cDNA (4.7 kb), and exon sizes have been found to range from 31 bp in exon 24 to 1889 bp in the 3′ terminal exon 27. A "hot spot" for recombination was predicted in the region of exons 13 to 17 on the basis of RFLP analysis of mutant RB genes (*35*).

Most retinoblastoma tumors express a full-length mRNA transcript and do not contain rearrangements detected by Northern (RNA) or Southern (DNA) blot analysis. Knowledge of the cDNA sequence and organization has been required to identify the mutations in retinoblastoma tumors. Some previously unidentified mutations have been detected by ribonuclease (RNase) protection to localize probable mutations, followed by polymerase chain reaction to amplify and then sequence the mutated region (*36*).

In a sample of tumors from small cell lung cancer and pulmonary carcinoids, large deletions and rearrangements in *RB* DNA were detected as well as loss of *RB* mRNA, whereas in non–small cell tumors of various sorts, no changes in *RB* were detected in DNA or in mRNA expression (*15*). Ultimately, a full molecular analysis of *RB* mutations will be needed to understand the range of mutational events that can lead to functional inactivation.

A decisive experiment in identifying a tumor suppressor gene is transmission of the suppressed phenotype by transfer of the wild-type gene into targeted tumor cells. In a recent report (*37*), the cloned cDNA (4.7 kb) was introduced by retroviral-mediated gene transfer into a retinoblastoma cell line, each containing a deleted *RB* gene and expressing neither the mRNA nor the protein. Clones of morphologically flat cells were detected among the neomycin-resistant transfectants that received the viral construct. Most of these clones later reverted to small cells resembling the uninfected parent, but eventually a few clones of stable flat cells were recovered. These cells grew slowly in culture, but not in soft agar nor in nude mice. They expressed the *RB* mRNA and protein. Thus, expression of the transfected *RB* gene led to regulated growth and loss of tumor-forming ability.

The nuclear protein, p53, was initially identified as a cellular encoded gene product that formed complexes with simian virus 40 (SV40) T antigen in SV40-transformed rodent cells (*38*) and was subsequently cloned (*39*). The finding that p53 protein is overexpressed in transformed rodent cells and tumors led to the view of the p53 gene as a positive effector in tumor formation (*40*). However, genetic evidence now shows that the regulated wild-type gene is a tumor suppressor (*41*). Wild-type p53 also plays a protective role against the transforming effects of the Friend erythroleukemia virus (*42*).

Mutant p53 protein has a transforming effect on primary rat cells, shown by its tumor-promoting activity in *ras*-transfected cells. In contrast, wild-type p53 transfected simultaneously with mutant p53 plus *ras* will suppress transformation (*41*). Mutant p53 proteins have been found complexed with the wild-type form in heterodimers, suggesting that the mutants exert their positive effect by diluting out the wild-type homodimers and conversely that the wild-type form can inhibit the transforming function of the mutant protein by the same process. The mutant proteins may have other functions in addition to sequestering the wild-type form (*41*). For example, transgenic mice carrying mutant p53 develop lung tumors (*43*). The protein p53 may be unique in that the wild-type allele is a suppressor, whereas some mutant alleles act as tumor promotors.

Genes that function as suppressors of *ras* were selected indirectly in cells with a flat revertant morphology. Noda and co-workers isolated a gene called K*rev-1* from a human fibroblast cDNA expression library that had been cloned into a *neo*-containing plasmid and transfected into v-K-*ras*–transformed NIH 3T3 cells. The *neo*-resistant transfectants were pooled and enriched for non-transformed clones by a series of negative selection procedures (*44*). Several transfectants were nontumorigenic in nude mice and grew very poorly in low serum or soft agar. Nonetheless, they contained several copies of K-*ras* and expressed the K-*ras* product, p21. Thus, the transforming ability of K-*ras* p21 oncoprotein was suppressed posttranslationally. The K*rev-1* human cDNA was cloned from one transfectant, sequenced, and shown to be structurally related to the *ras* gene family. R16 cells containing K*rev-1* resist retransformation by v-K-*ras*, but can be retransformed by v-*src*, v-*mos*, v-*raf*, or v-*fos*. These and other results suggest that K*rev-1* competes with p21 rather than suppressing transformation induced by other oncogenes in NIH 3T3 cells.

Recently, a set of guanosine triphosphate (GTP)–binding proteins were isolated from bovine brain membranes, purified, and characterized (*45*). One of them is identical to K*rev-1*. The set includes tissue-specific *ras*-like proteins and reveals a complex array of factors that regulate GTP-associated events in different tissues.

Schaefer *et al.* (*46*) cotransfected high molecular weight genomic DNA from human placenta together with a plasmid containing a selectable marker (hygromycin resistance) into preneoplastic rat cells that had been previously transfected with human H-*ras* (EJ-*ras*). Transfected clones with a flat morphology were selected. They still contained the EJ-*ras* gene and produced its p21-encoded protein. An 18-kb Bam HI fragment was cloned and shown by retransfection to confer a flat morphology, anchorage dependence, and reduced tumorigenicity.

Kuchino *et al.* (*47*) found that transcription of c-*myc* was activated by Rous sarcoma virus infection of rat cells. In further studies of the virally infected cells, a new *myc*-related gene, S-*myc*, was identified. S-*myc* encodes a 47-kD protein related to mouse N-*myc* protein but lacking the acidic domain in the NH_2-terminal region. The protein expressed by the cloned S-*myc* gene suppressed tumorigenicity of rat RT4-AC tumor cells in nude mice. Evidence that mutant alleles of the same gene may encode either an oncogenic or a suppressive protein is also seen in studies of p53 discussed above.

Subtractive hybridization provides a general method for recovering genes that are expressed in normal cells but not in closely related tumors (*48*). Single-stranded cDNA from the normal cells is hybridized with a large excess of mRNA from the tumor cells to induce formation of double-stranded DNA-RNA hybrids of all messages produced by both parents. Unpaired cDNAs are recovered from hydroxyapatite columns to which the hybrid molecules bind and are used as probes to recover full-length cDNAs from an appropriate library. In principle, the method makes it possible to recover any expressed gene, although rare transcripts require special techniques for recovery. Nonetheless, subtraction is a relatively easy method for

recovering putative suppressor genes; a sticking point has been the choice of closely related normal and tumor cell populations.

Our laboratory has developed methods to use human breast tumor cells obtained from surgery for subtraction against normal breast epithelial cells from the same patient (*49*). At the outset, no suitable medium was available in which both normal and tumor cells could be grown, so that differential mRNA expression would not reflect different conditions of growth; and primary tumor cells had rarely been grown in long-term culture. A medium was devised, DFCI-1, which supports the long-term growth of normal and tumor breast epithelial cells.

Three clones of interest were recovered from subtractive hybridization with breast cells (*50*). These genes are expressed by all normal breast epithelial cells tested, but not by any of the primary tumors or tumor cell lines. One is keratin 5 (K5), encoding a structural protein expressed in normal breast epithelial cells in culture, but not in any primary tumor cells or cell lines in culture. When the normal cells are immortalized, K5 protein is expressed at a low level (*51*). Thus, K5 is a valuable marker to distinguish normal and primary tumor cells in culture, and raises the question whether K5 may have a regulatory as well as a structural function. Similarly, fibronectin is expressed in the normal cells but not in tumor lines, providing another marker to distinguish the two cell types. A third gene, NB-1, is not yet identified.

Suppressor genes are also found in invertebrates. In *Drosophila*, recessive mutations in a series of regulatory genes result in uncontrolled and invasive cell proliferation and death (*52*). One of these, the *lethal (2) giant larvae* gene has been cloned and the lethal phenotype shown to result from a lack of gene function. Tumorigenesis was prevented by reintegration of the normal gene into files that were deficient at this locus.

Functions of Tumor Suppressor Genes

Chromosome stability. Chromosome aberrations are the signature of the cancer cell. Tumorigenesis is driven by a cascade of genomic changes that engender the altered expression of cancer-related genes. These genomic changes include not only classical point mutations but, importantly, deletions that remove entire genes or disrupt their regulatory sequences, as well as translocations, amplifications, and other rearrangements. Analysis of LOH in retinoblastoma has implicated mitotic nondisjunction and recombination as well as physical loss (*18*). Substantial decreases in cytosine methylation, typically seen in many tumor types, may alter expression of many genes (*26*). However, Vogelstein and co-workers have speculated that, in addition, demethylation may contribute to chromosome instability by increasing chromosome stickiness (*26*).

The genes that regulate DNA repair could fit the criteria of tumor suppressors. Chromosome instability might be a direct consequence of the loss of tumor suppressor gene function. Effective high-fidelity DNA repair enzymes are major contributors to chromosome stability. Chromosomes undergo continual breakage and repair events, both spontaneous and environmentally induced. In cancer-prone recessively inherited diseases such as xeroderma pigmentosum, Fanconi's anemia, ataxis telegiectasia, and Bloom's syndrome, damaged DNA is improperly repaired. In Bloom's syndrome, for example, a mildly defective DNA ligase has been identified (*53*); and elevated frequencies of sister-chromatid and chromosome exchanges occur (*44*), suggesting derangement of this control as well. Although few mammalian enzymes have yet been associated with DNA repair processes, it is evident from studies with bacterial and yeast DNA repair-defective mutants that many enzymes are involved.

Differentiation and senescence. Differentiation is clearly a mode of tumor suppression, since terminally differentiated cells lose the ability to divide. However, most tumors consist of partially differentiated cells that can be recognized as liver, pancreas, lung, breast, and so on, and yet they proliferate. The real issue lies in understanding how the differentiation pathways are regulated and how they are blocked in neoplasia. At what stage does differentiation become an alternative to proliferation (*55*)?

In suppressed N × T hybrids formed by fusion between cells of different differentiation pathways, the hybrids may express the differentiated state of the normal rather than the tumor cell type (*7*). This intriguing observation suggests that the expression of tumor suppressor genes in the normal parent may be linked to their differentiation pathway, or even that they may be one and the same. A relation between the RB gene and differentiation has been suggested by evidence that the RB gene remains in the underphosphorylated state in differentiating cells of various types (*56*). Cyclic phosphorylation of RB is involved in cell cycle regulation as discussed below.

Transfection with oncogenes such as *myc* can block differentiation and promote proliferation of cells at particular stages of development but not others [see (*6*) for discussion]. Studies with *myc* (*6*) illustrate how normally regulated interactions in development can be upset or deregulated by oncogenes, but also that oncogenes can drive proliferation and block differentiation only at specific stages in the differentiation pathway.

Some light has been cast on this complex issue in experiments involving senescence, which is a specific kind of differentiation. In these experiments, normal human fibroblasts were transformed with v-K-*ras* (*57*). If the cells had been previously immortalized (by SV40 viral infection and long-term selection in culture), the K-*ras* transformants made large tumors in nude mice, but if not, the transformants made microtumors and then senesced. The role of senescence as a mechanism of antitumor protection in vivo is debatable, but evidence that senescence is a dominant trait (*58*) emphasizes the importance of mutations that confer indefinite life-span as an essential step in mammalian tumorigenesis.

The discovery that two suppressor genes, *RB* and p53, are intimately involved in cell cycle regulation has begun to clarify aspects of the molecular basis of senescence. The immortalizing function of DNA tumor virus gene products that form complexes with *RB* and p53 will be discussed below.

Control of cell proliferation. A key role of tumor suppressor genes is in the inhibition of cell proliferation. The regulation of cell proliferation pathways involves growth factor receptors, signal transduction pathways from membrane to nucleus, and nuclear binding proteins that regulate transcription as well as posttranscriptional controls. Until recently, research in this area has focused on genes that facilitate proliferation. These genes, the oncogenes, were largely identified as mutant cellular homologs of retroviral-transformed genes, in which the mutations were gain-of-function changes (*9*). The wild-type cellular alleles of retroviral oncogenes (proto-oncogenes) contribute to homeostasis by maintaining normal regulatory controls, but these controls are lost in the oncogenic mutant alleles. Because the wild-type gene product regulates proliferation and the altered or overexpressed form induces unregulated growth, these mutations have been called dominant.

Two recently cloned genes, *RB* and p53, are the first examples of tumor suppressors that may function in the control cell proliferation. Since mutations in these genes are being found in many different tumors, *RB* and p53 are of great potential value in illuminating mechanisms of suppressor gene function.

The RB protein is a 110-kD phosphoprotein (*59*), which undergoes additional phosphorylation in the cell cycle (seen in gels as

bands of slightly lower mobility, that is, 112 to 114 kD) (*60*). These over-phosphorylated forms are not seen in resting cells, but they appear as cells approach the G_1/S boundary in the cell cycle, and are present throughout S and G_2 phases (*56, 60*). The overall amount of RB protein does not change, but the phosphorylation of RB undergoes rapid turnover. Only a few of the many phosphorylated serines on the protein are involved in this recycling. The RB protein forms complexes with SV40 T antigen, the transforming protein of SV40 virus (*60*); adenovirus E1A protein, which is required for adenovirus-induced cell transformation (*61*); and the E7 protein of human papilloma virus (HPV) (*62*), which is required for HPV-induced tumor formation. An amino acid sequence comparison has identified two domains in E1A, involved in binding to the RB proteins that show sequence similarity with domains encoded by E7 and SV40 large T (*62*).

SV40 T antigen binds to the basal protein, $p110^{RB}$, but not to the over-phosphorylated forms ($pp112^{RB}$ to $pp114^{RB}$), whereas E1A binds to both (*60*). In virally transformed cells, the binding of RB to T antigen, E1A, or E7 contributes to unregulated growth by removing RB from its normal inhibitory role. As yet, these viral-RB complexes have only been seen in in vitro studies (*56, 60–62*). In retinoblastomas, the RB protein is either absent or altered by mutation in ways that presumably alter its complexing ability and thereby interfere with its inhibitory function. On the basis of these data and inferences, it has been postulated that the *RB* gene, which is expressed in most or all normal cell types, acts as an inhibitor of cell cycle progression, and that the inhibition is released by phosphorylation in normal cells and in virally infected or tumor cells by competitive binding of the inhibitory $pp110^{RB}$ protein, thereby interfering with its normal inhibitory role.

A totally unexpected result of the recent *RB* and p53 studies is the likelihood that their functions are coupled in joint effects on regulation of the cell cycle. Coprecipitation studies have shown similarities in binding patterns: both RB and p53 protein bind to SV40 T antigen, but in different domains (*41, 60*). In an analogous manner, E1A and E1B, the transforming proteins of adenovirus, bind respectively to RB and p53 protein (*39, 61*). In parallel studies with HPV, it has been shown that *E7* binds to *RB* (*62*). *E6* has been identified as a viral oncogene (*63*), and it will be important to establish whether p53 is a cellular target of *E6* and to what extent it acts in a manner similar to E1B and T antigen.

These in vitro data do not stand alone; rather they strengthen and confirm inferences from a variety of studies of virally induced immortalization of rodent and human cells. While much remains to be worked out, the correlation of these studies with the widespread occurrence of *RB* and p53 mutations in human tumors further strengthens the view that these genes play a central role in tumor suppression.

The tumor-suppressing effects of senescence were discussed earlier. Neither SV40 nor adenovirus immortalize human cells reproducibly, although rare instances have been reported. On the contrary, HPV does immortalize normal human keratinocytes in culture consistently (*64*), and the *E6* plus *E7* genes of HPV type 16 are necessary and sufficient for this process (*63*). In recent studies, HPVs have been associated with squamous carcinomas, especially cervical carcinoma, which are derived from squamous epithelial cells, for example, keratinocytes. In our laboratory, normal human breast epithelial cells have been immortalized by HPV types 16 and 18 (*65*). Since HPV has not been associated with breast cancer, this result raises the possibility that HPV may be involved in breast or other tumors of epithelial origin. That HPV succeeds when no other related DNA tumor viruses are effective in immortalizing human cells suggests differences in the detailed modes of viral protein interactions with cellular targets.

Tumor Suppression Is the Norm

Among the mysteries of cancer, two stand out. One is the age incidence of cancer, and the other, perhaps related to it, is the long and variable time course of progression in the adult solid tumors. Cancer primarily is a disease of late life. Thus, during much of our lives, we are protected against cancer, despite the presence of some 10^{14} potentially tumorigenic cells in our bodies. Contrast this situation with rodents, which are susceptible to cancer in the short 1 to 2 years of their lives. Evolutionary mechanisms must have developed to protect us long-lived creatures at least throughout our reproductive period.

But neither do all rodents die of cancer. Thus, anticancer mechanisms must already be in play throughout evolution; even *Drosophila* has recessive mutations leading to lethal tumors (*35*). Genes such as p53 appear to have similar modes of action in rodents and humans. On the contrary, no *RB* mutants are known in lower mammals.

My proposal here is that tumor suppressor genes play a key role in cancer protection. Some mechanisms are common to rodents and humans, whereas others have undergone evolutionary development. The development may take the form of attenuating the effectiveness of each step toward tumor formation or requiring more steps. Thus, we should anticipate sequence and function similarities in some cancer-related genes, but not in all.

Tumor suppressor genes provide a vast untapped resource for anticancer therapy. They represent nature's own approach to protection against cancer. To find which genes will be most effective, either at the protein level or eventually in gene therapy, is the puzzle and the promise of tumor suppressor genes.

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Research Article

The Cholinergic Neuronal Differentiation Factor from Heart Cells Is Identical to Leukemia Inhibitory Factor

TETSUO YAMAMORI, KEIKO FUKADA, RUEDI AEBERSOLD, SIGRUN KORSCHING, MING-JI FANN, PAUL H. PATTERSON*

A protein secreted by cultured rat heart cells can direct the choice of neurotransmitter phenotype made by cultured rat sympathetic neurons. Structural analysis and biological assays demonstrated that this protein is identical to a protein that regulates the growth and differentiation of embryonic stem cells and myeloid cells, and that stimulates bone remodeling and acute-phase protein synthesis in hepatocytes. This protein has been termed D factor, DIA, DIF, DRF, HSFIII, and LIF. Thus, this cytokine, like IL-6 and TGFβ, regulates growth and differentiation in the embryo and in the adult in many tissues, now including the nervous system.

A GROUP OF PROTEINS, OFTEN CALLED CYTOKINES, REGULATE growth and differentiation in a wide variety of tissues, both in the embryo and in the adult organism. Some of these proteins, such as interleukin-6 (IL-6), were first recognized for their effects on myeloid cells. The generation of the diverse array of myeloid cells is under the control of cytokines and proteins termed

The authors are or were with the Biology Division, California Institute of Technology, Pasadena, CA 91125. Present addresses: R. Aebersold, Biomedical Research Center, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5; K. Fukada, Department of Anatomy and Cell Biology, SUNY Health Science Center, Brooklyn, NY 11203; S. Korsching, Max-Planck-Institut für Entwicklungsbiologie, Abteilung Physikallische Biologie, D-7400 Tubingen 1, Federal Republic of Germany.

*To whom correspondence should be addressed.