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The current state of oncogenes and cancer: Experimental approaches for analyzing oncogenetic events in human cancer

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Abstract

The development of cancer is a multistage process. The activation of proto-oncogenes and the inactivation of tumor suppressor genes play a critical role in the induction of tumors. Using human cell model systems of carcinogenesis, we have studied how oncogenes, tumor suppressor genes, and recessive cancer susceptibility genes participate in this multistep process. Normal human cells are resistant to the transforming potential of oncogenes, such as *ras* oncogenes, which are activated by specific point mutations. Since as many as 40% of some tumor types contain activated *ras* oncogenes, a preneoplastic transition in multistage carcinogenesis must involve changing from an oncogene-resistant stage to an oncogene-susceptible stage. The analysis of such critical steps in carcinogenesis using rodent systems has usually not represented the human disease with fidelity. In order to study this carcinogenic process, we have developed human cell, *in vitro* systems that represent some of the genetic changes that occur in cellular genes during human carcinogenesis. Using these systems, we have learned some of the functions of dominant activated-transforming oncogenes, tumor suppressor genes, and cellular immortalization genes and how they influence the carcinogenic process in human cells. Using our understanding of these processes, we are attempting to clone critical genes involved in the etiology of familial cancers. These investigations may help us to develop procedures that allow us to predict, in these cancer families, which individuals are at high risk for developing cancer.

Genetic events in multistage carcinogenesis

It is well established that the development of cancer (carcinogenesis) requires multiple steps [1, 2]. Epidemiological studies, age-dependent tumor incidence, histopathology studies, and experimental models have indicated the multistep nature of tumor induction and progression [3–5]. The concept of a genetic basis for cancer dates back to the turn of the century, when Boveri contended that chromosomal changes play a major role in the cancer process. Support for this notion was enhanced by the discovery of cellular oncogenes, and these genes have formed the basis for our understanding of the genetic events in cancer [6–9]. When normal proto-oncogenes become activated to potential tumorigenic oncogenes by structural and regulatory alterations, they can play a direct role in tumorigenesis. It is evident that cells of all vertebrates and invertebrates as diverse as man, fish, frog, and *Drosophila,* as well as lower single-cell eukaryotes such as yeast, contain proto-oncogenes. These genes possess a high degree of interspecies homology even in totally unrelated species [10]. Certain proto-oncogenes are transcribed in particular cell types at specific times during normal embryogenesis, and transiently when cells are stimulated by mitogens to proliferate [11, 12]. Alterations affect-

Table 1. Proto-oncogenes

Proto-oncogenes	Possible function	Location on human chromosome
Growth factor		
EGF	Ligand	4q25
TGF alpha	Ligand	2p
TGF beta	Ligand	19q
PDGF	Ligand	22q
FGF	Ligand	?
CSF-1	Ligand	5q
Trans membrane tyrosine kinase		
c-*erb* B-1	Receptor for EGF & TGFalpha	7p11.2
c-*erb* B-2	Receptor for ?	17q11.2
c-*fms*	Receptor for CSF-1	5q33
met	Receptor for ?	7q
ros-1	Receptor for ?	6q
kit	Receptor for ?	4q13
trk	Receptor for ?	1q32.1
tyk-1	Receptor for ?	?
tyk-2	Receptor for ?	?
Cytoplasmic		
c-*abl*	Protein tyrosine kinase	9q34.1
c-*src*	Protein tyrosine kinase	20q13.3
c-*fes*/*fps*	Protein tyrosine kinase	15.26.1
c-*fgr*	Protein tyrosine kinase	1p36.1
c-*yes*	Protein tyrosine kinase	18q
c-*fyn*	Protein tyrosine kinase	?
c-*lyc*	Protein tyrosine kinase	?
c-*lck*	Protein tyrosine kinase	1p
c-*tkl*	Protein tyrosine kinase	?
hck	Protein tyrosine kinase	20q
c-*mos*	Serine/threonine kinase	8q
raf 1,2	Serine/threonine kinase	3p25, 4
rsk	Serine/threonine kinase	?
crk	PI specific phospholipase C	?
G-protein like		
H-*ras*	GTP binding, GTPase	11p15
K-*ras*	GTP binding, GTPase	12p12-pter
N-*ras*	GTP binding, GTPase	1p13.1-p21
Nuclear		
c-*myc*	DNA binding	8q24
N-*myc*	DNA binding	2p24.2
c-*fos*	Transcription factor	14q
c-*jun*	Transcription factor	1p
c-*myb*	Transcription factor	6q
c-*rel*	Transactivation	3
ski	Transcription factor	1q
sno	Related to ski	?
ets 1,2	Transcription factor	11q, 21q
erg	Related to ets	?
erb A 1,2	Hormone receptor transcriptional control	17q, 3p

ing the expression or function of proto-oncogenes are widely considered to be contributing causes for cancer development [13]. It is thought that groups of functionally diverse proto-oncogenes play a critical role, perhaps cooperatively, in governing normal cellular proliferation and/or differentiation by functioning at distinct steps in intracellular signal transduction of the growth factor cascades.

Proto-oncogenes can be classified into groups by the location and biological activity of their products: secreted growth factors, cell surface receptors with the associated protein kinases activity, cytoplasmic protein kinase, and nuclear proteins with transcription factor activity. For example, the c-*sis* gene product has been identified as the beta-subunit of platelet-derived growth factor (PDGF) [14]; the products of c-*erb* B and c-*fms* genes have been identified as the cell surface receptors of epidermal growth factor (EGF) and monocyte colony stimulating factor 1, respectively [15, 16]; and the *erb* A protein has been identified as a T3 thyroid hormone nuclear receptor [17]. It has been demonstrated that growth factors, like PDGF, are able to induce expression of nuclear proto-oncogenes c-*myc,* c-*fos,* and c-*jun* [18–20]; c-*mos* is able to induce mitotic maturation [21]. From these results and others, proto-oncogene protein products seem to be involved in many steps of the growth factor receptor mediated intracellular signaling pathway.

However, there is no evidence that the activation of a single cellular oncogene, such as *ras* oncogene, can change a normal diploid cell into a tumor cell. Numerous experiments indicate that the tumorigenic process requires the activation of certain oncogene(s) as well as the loss of a second class of genes called tumor suppressor genes or recessive cancer susceptibility genes.

This need for multiple genetic alterations for tumorigenic conversion of normal cells has also been shown by transfection experiments into diploid embryonic fibroblasts [22]. In contrast to the already immortalized and aneuploid NIH/3T3 cells, cultures of this embryonic fibroblast cell can be transformed only if the activated *ras* gene is co-transfected with constitutively expressed c-*myc* or, alternatively, with one of the DNA viral T antigens such as SV40-LT or adenoviral E1a genes [22, 23]. Transgenic mice carrying *myc* gene constructs linked to an Ig enhancer develop pre B or B cell lymphomas that are mono- or bicolonal and appear only after a considerable latency period, indicating that one or several additional events must contribute to tumor development [24]. The activation of a second oncogene or inactivation of a tumor suppressor gene might be suggested as a further alteration. The progression of Burkitt's lymphoma requires at least three identifiable steps: infection with Epstein-Barr virus enhances the growth of a subset of B cells; this is followed by a translocation of c-*myc* locus on chromosome 8 and then the possible activation of a *ras* oncogene [13].

Several lines of evidence point to the existence of a class of genetic elements termed tumor suppressor genes or recessive cancer susceptibility genes. When they become homozygously inactivated by deletion or point mutation a tumor can arise. This class of gene was predicted by Knudson and Strong in the 'Two-Hit' hypothesis, in which they stated that cancer will occur only after the loss of two alleles at a particular genetic locus [25]. According to their theory, both genetic events take place in somatic cells in sporadic cancer, whereas in inherited cancers one of the genetic hits takes place in the germ line and the second occurs in somatic cells.

Harris and coworkers [26] found that somatic cell hybridization of normal and malignant rodent cells results in the suppression of the tumorigenic phenotype. The reappearance of tumorigenicity is accompanied by chromosome loss. The loss of chromosomes 1 and 4 from nontumorigenic somatic cell hybrids of human fibroblasts and HT1080 fibrosarcoma cells has been shown to correlate with the re-emergence of tumorigenicity [27]. The involvement of a normal chromosome in the suppression of tumorigenicity was further demonstrated by transferring a normal chromosome into the tumor cells. For example, the introduction of normal human chromosome 11 into a Wilms' tumor cell line suppressed the ability of the cell to induce tumors in nude mice [28]. This provided direct evidence for the involvement of chromosome 11 in the suppression of tumorigenesis. This has also been demonstrated by transferring a single chromosome 11 into

Hela cells]29], although different regions of chromosome 11 are believed to be involved.

Another line of evidence for the existence of tumor suppressor genes has been the finding that certain chromosomal loci undergo a nonrandom loss of heterozygosity in particular human tumors [30] (see Table 2 and references therein). By comparing the restriction fragment length polymorphism (RFLP) in normal and tumor tissue, many laboratories have documented the tumor specific loss of one parental allele of a polymorphic DNA marker [30]. These results were consistent with the loss of tumor suppressor loci, and, with the cloning of the retinoblastoma susceptibility locus, this supposition was confirmed [31].

Morphological and nontumorigenic revertants can be isolated from *ras* transformed rodent cells. Krev-1, a *ras* related gene that has the ability to suppress *ras* transformation, was isolated from revertants of Kirsten sarcoma virus-transformed NIH/3T3 cells by transfecting with DNA from a cDNA expression library made from the RNA of a normal human fibroblast [32, 33]. Schaefer *et al.* [34] were also able to transfect tumor suppressor activity from human placental DNA into similar cells, obtaining suppressed cell lines from which they cloned a DNA segment with tumor-suppressing activity. There is reason to believe that such genes may mediate growth- inhibiting processes such as differentiation.

Differentiation of human teratocarcinoma cells [35] and murine myocytes [36] can be blocked by the expression of an activated *ras* oncogene. Differentiation can also be blocked by the expression of a temperature-sensitive oncogene. For example, it has been shown that myoblasts transformed by a

Table 2. Loss of alleles associated with human malignancies

Tumor	Chromosome location	Reference
Neuroblastoma	1p	122, 123
Ductal breast carcinoma	1p, 13q	124
Uveal melanoma	2	125
Small-cell lung carcinoma	3p, 13q	126
Squamous cell and large-cell carcinoma	3, 11, 13, 17	127
Adenocarcinoma of the lung	3p	126
Renal cell carcinoma	3p	128
Von Hippel-Lindau disease	3p	129
Colorectal carcinoma	5p, 17p, 18q	130
Familial polyposis coli	5q	131
Salivary gland tymor	6	132
Acute lymphoblastic leukemia	9p21–22	133
Non-Hodgkin's lymphoma	9p21–22	133
Multiple endocrine neoplasia type 2A	10	134
Glioblastoma	10	135
Multiple endocrine neoplasia type 1	11q	136
Wilms' tumor	11p	137
Hepatoblastoma	11p	138
Rhabdomyasarcoma	11p	138
Bladder carcinoma	11p	139
Cervical tumor	11	140
Breast carcinoma	11p, 13q	141, 142
Retinoblastoma	13q	143
Osteosarcoma	13q	144
Soft tissue sarcomas	13q	145
Acoustic neuroma	22	146
Meningioma	22	147
Bilateral acoustic neurofibromatosis	22	148

temperature-sensitive Rous sarcoma virus mutant failed to differentiate at permissive temperature but could form differentiated myotubes at nonpermissive temperatures, and subsequent reexpression of transforming protein could no longer prevent the maturation process [37]. The expression of chondrocyte and melanoblast differentiation markers was also prevented by a temperature-sensitive Rous sarcoma virus at permissive temperature. These differentiation markers reappeared after a short incubation at nonpermissive temperature. If these cells were kept at the nonpermissive temperature for a sufficiently long time, they lost the ability to become transformed [38, 39]. Murine teratomas can differentiate normally in the appropriate early embryonic environment and can generate a full range of normal mouse tissues [40]. These data suggest that activated oncogenes can only transform cells at certain stages at which they can prevent further differentiation.

Deletion or mutational inactivation of tumor suppressor genes has been shown to play an important role in the development of retinoblastoma, oesteosarcoma, mammary carcinoma, and some other tumor types [30]. These tumor cells often lack a functional Rb-1 protein. When the wild-type Rb-1 gene was introduced into retinoblastoma cells, the ability of these cells to form tumors was suppressed, probably due to severe growth inhibition [41]. Recent data suggest that the Rb-1 gene protein product plays a critical role in controlling the cell cycle [42, 43]. The binding of the Rb protein to SV40 T-antigen appears to be essential to the mechanism by which that virus transforms cells [44, 45]. Other DNA viruses with the ability to immortalize cells interact with the Rb-1 protein [46]. Interestingly, p53, a putative tumor suppressor gene, was first identified by its binding to the SV40 T-antigen protein [47–49], and it is believed that immortalization of cells in culture requires an inactivated p53 gene. The Rb-1 protein is phosphorylated in a cell-cycle dependent manner by CDC-2, a protein kinase that is active at certain times in the cell cycle [50]. The nature of the oncogenes active in tumors that lack a functional Rb-1 gene is unclear, although N-myc amplification, additional copies of chromosome 1, and isochromosome 6p are often observed in retinoblastoma tumors [51, 51].

The involvement of oncogenes in human neoplasia

A variety of proto-oncogenes have been found to be frequently altered in human tumors, and clinical correlations of the involvement of certain oncogenes exist for several tumor types. Table 3 lists some well-documented examples; some of them are discussed below.

In several human tumor types, the *ras* family of genes is frequently found to be activated by a single point mutation. This family consists of three genes:

Table 3. Proto-oncogene alteration in human malignancy

Proto-oncogene	Activation	Tumor
c-*myc*	Amp.	SCLC, breast and colon carcinoma
c-*myc*	Chrm. translocation	B and T cell lymphoma
c-*abl*	Chrm. translocation	CML
N-*myc*	Amplification	Neuroblastoma, retinoblastoma
c-*erb* B	Amplification	Squamous carcinoma
c-*neu*	Amplification and overexpression	Adenocarcinoma of breast
N-*ras*	Point mutation	AML, ALL, MDS, melamonas, some carcinomas and sarcoma
K-*ras*	Amplification and point mutation	Colon, lung and bladder carcinoma, pancreatic carcinoma
H-*ras*	Point mutation	Carcinoma and melanomas
bcl-1	Chrm. translocation	B cell lymphoma
bcl-2	Chrm. translocation	Follicular lymphoma

K-*ras*, H-*ras*, and N-*ras* [53]. The protein products of these *ras* genes are very similar, with a molecular weight of 21,000 daltons. *Ras* proteins become activated by binding GTP upon stimulation, and transduce signals to some still undefined effector proteins. Mutationally activated *ras* gene proteins have a point mutation at codons 12, 13, or 61, which inactivates its GTPase activity, leaving the protein in a constitutively activated state. The functional and structural similarities between the p21 *ras* proteins and G proteins controlling adenylate cyclase suggest that normal p21 *ras* protein is involved in the transduction of external stimuli induced by growth factors. A protein has been found to be involved in the hydrolysis of the GTP bound to *ras* protein. The protein, GTPase-activating protein (GAP), binds to the effector domain of the *ras* protein, and might play a role in signal transduction [54–56]. *Ras* genes were first identified as the transforming genes in the Harvey and Kirsten sarcoma viruses. Mutated *ras* genes were first isolated from human tumors because of their ability to transform NIH/3T3 cells by transfection of DNA extracted from human tumor cells [57, 58]. Subsequent analysis revealed that *ras* gene mutations can be found in a variety of tumor types [59]. In colon carcinoma tissues and in the large villous types of adenomas of the colon, the K-*ras* gene is the predominantly mutated form of the *ras* gene, and has been detected in approximately one-half of the cases analyzed by Bos et al. [60]. The loss of genetic information has also been observed in colon cancer development. Vogelstein and coworkers have demonstrated the temporal involvement of at least four genetic events in colon cancer development [61]: the loss of heterozygosity of alleles on chromosome 5p, the activation by point mutation of the K-*ras* oncogene on chromosome 12, the inactivation of the p53 locus on chromosome 17, and the inactivation of a gene on chromosome 18. In non-small cell lung carcinomas, including adenoma, squamous, or epidermoid cell carcinoma, and in large cell carcinoma, *ras* mutations are only found in adenocarcinoma. The incidence is about 30%, and nearly all are point mutations at K-*ras* gene codon 12 [62, 63]. Transgenic mice, carrying a plasmid that contains a mutated H-*ras* oncogene driven by an SV40 promoter or immunoglobulin enhancer, express the *ras* gene predominantly in lung and developed multicentric adenomatous tumors comparable to the well-differentiated adenocarcinoma of the lung in humans [64]. This experiment indicates that the activated *ras* gene plays a critical role in the formation of this tumor and the power of transgenic mouse experiments.

The incidence of *ras* mutations is about 20% in human melanoma [59]. Most of the mutations are found in the N-*ras* genes occurring at various stages of tumor development. Altough the metastatic properties of melanoma do not seem to be influenced by the presence or absence of an activated N-*ras* gene, there is a strong correlation between the presence of an activated *ras* gene and primary tumor location at a sun-exposed site [65, 66]. A mutated K-*ras* oncogene can be found in 90% of human exocrine pancreas tumor, and all of the mutations were located in codon 12 of the K-*ras* oncogene [67]. In thyroid carcinomas, activation of all three *ras* genes has been found in the benign follicular adenomas and in 50% of the cases of the follicular undifferentiated carcinoma [68]. Mutations in the N-*ras* gene, K-*ras* gene, and, less frequently, in the H-*ras* gene were detected mostly in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) in about one-third of the cases studied [69, 70]. The presence of *ras* mutation in MDS and AML might have some direct clinical relevance. MDS patients with *ras* gene mutations may have a higher chance of progressing into AML, and so may have a poorer prognosis [70]. Several studies suggest that the prognostic value of the *ras* mutation may be limited [71]: the poorer prognosis may be due to the effects of the *ras* oncogene on the tumor cells. It has been reported that cells containing an activated *ras* gene coming from a multipotent stem cell appear to be more resistant to chemotherapy than *ras* mutation- containing cells occurring later in the course of MDS and AML [72]. Therefore, the *ras* mutation might be a suitable marker for monitoring the effect of chemotherapy and detecting minimal residual disease.

Most Burkitt's lymphoma cells have a c-*myc*/Ig juxtaposition by a chromosomal translocation in-

volving chromosome 8 at band q24, the site of the c-*myc* proto-oncogene. The reciprocal site is chromosome 14q32, the immunoglobulin heavy chain (IgH) locus [73, 74]. The 8 : 14 marker is the most commonly found in Burkitt's lymphoma (about 90% of cases); the remaining 10% contain c-*myc* translocations to chromosome 2 at band p12 or chromosome 22 at band q11, the sites of the kappa and lambda light chain genes, respectively. Similar chromosomal translocations have been seen in most cases of B cell acute lymphoblastic leukemia (B-ALL) and in some pre-B-ALL cases, both of which are non-Burkitt's B cell lymphomas [75]. Some oncogenes are altered in a wide variety of tumor types, whereas others are activated only in specific types of tumors. For example, c-*myc* is amplified in diverse carcinomas and sarcomas and in highly malignant plasma cell leukemias. The approximate frequency of amplification of c-*myc* in these tumors is 10%. N-*myc* amplification and overexpression is restricted to neuroendocrine tumors and appears in stage III and IV neuroblastomas [76]. It is thought that the amplification of N-*myc* in neuroblastoma may be related to tumor progression. The involvement of the c-*myc* oncogene expression has been analyzed in head and neck tumors, and elevated c-*myc* gene expression was correlated with poorer prognosis in head and neck squamous cell carcinomas [77].

Chronic myelocytic leukemia (CML) is a disease of pluripotent hematopoietic stem cells with biphasic and triphasic clinical courses [78]. More than 90% of such patients have the Philadelphia chromosome, a reciprocal translocation involving the long arms of chromosomes 9 and 22 [79]. This structural alteration produces a fusion protein encoded by the 3' end of the chromosome 22 associated sequences bcr and transposed oncogene c-*abl*, leading to the expression of an abnormal protein that coexists with its normal c-*abl* protein [80]. The *bcr-abl* protein differs from c-*abl* not only in size but also in the fact that it has an abnormal tyrosine kinase activity, similar to that of the protein product of transforming v-*abl* gene [81]. In cases of CML in which the Ph' chromosome is lacking, there is a molecular rearrangement of the c-*abl* gene. Molecular probes often detect the rearrangement in these cases, whereas karyotype analyses for Ph' chromosome are negative, indicating an unusual translocation of *bcr* and *abl* [78]. The Ph' chromosome also occurs in 25% to 30% of adult cases and in 2% to 10% of childhood cases of ALL. Their leukemia cells express a novel c-*abl* tyrosine kinase (185–190 kd) as a result of the fusion of the putative first exon of the *bcr* gene with *abl*. These molecular events may be used as markers in confirming the diagnosis of CML and related diseases [78].

The *neu* oncogene appears to encode a p185 growth factor receptor that shares structural similarities with the EGF receptor, and its associated tyrosine kinase appears to be essential for the transforming activity of the *neu* oncogene [82]. This putative receptor can be activated to become a transforming oncogene by a single point mutation in its transmembrane domain or by overexpression [83]. Amplification or overexpression of the *neu* gene has been reported in 30% of human breast cancer cells [84, 85]. A significant correlation has also been reported between *neu* gene amplification and patients with lymph node involvement in this disease [85, 86]. In addition, the amplification or overexpression of the *neu* oncogene is a significant predictor of both overall survival and time to relapse in patients with breast cancer, and it has greater prognostic value in lymph-node negative disease than most currently used prognostic factors, such as estrogen receptor level.

In vitro cell models for analyzing progression toward tumorigenicity

It has been difficult to prove a causal role for most of the genetic elements described here in human neoplasia. In order to prove a statistically significant correlation for the role of an oncogene or suppressor gene at some stage of human cancer, large numbers of samples must be analyzed. This type of large-scale retrospective study can involve more than one thousand tumor samples [85], and often the conclusions remain controversial. Therefore, we have taken the approach of establishing cell culture systems to study the mechanisms of

oncogene-induced transformation of human cells. Human cells in culture are inherently more stable than rodent cells, and represent a controllable means of studying the interaction of oncogenes and tumor suppressor genes. They also provide a means of detecting new genetic elements that are involved in human cancer.

In vitro experimental systems that address multiple stages of the carcinogenic process have been difficult to develop because of the complexity of the genetic changes that occur during oncogenesis. The long-term objectives of establishing such systems have been to develop cell lines dependent on multiple genetic events for the induction of tumorigenesis, to characterize those genes and their effects on the biology of human cells, and to use the cell lines developed to isolate new oncogenes from human tumors or human suppressor genes that effect a transition in the multistep carcinogenic process.

Until recently, studies of the biological effects of human oncogenes were limited to experiments with rodent cell lines such as NIH-3T3. These cells are nontumorigenic, but are far removed from normal. They can be considered to be on the verge of tumorigenicity, requiring only a single additional genetic event to gain the capacity to form tumors. In those systems the genes from human tumors that had biological activity were very often found to be members of the *ras* family of oncogenes [87–94]. However, the vast majority of DNA samples from human tumors do not have transforming activity in the NIH-3T3 cell assay.

A more relevant system for studying multiple genetic changes during oncogenesis has been that of primary rat embryo fibroblasts. It has been suggested that tumorigenic transformation of rat embryo cells requires the cooperation of genes that can be grouped into two classes [22, 23]. The first group of oncogenes is required to rescue cells from senescence, while the second group actually triggers the tumorigenic phenotype. The first group's gene products (c-*myc*, L-*myc*, N-*myc*, p53, *myb*, Adeno E1a, polyoma LT, SV40LT, papillomavirus E7) reside in the nucleus and render the cells competent for transformation by the second group of genes, whose products (H-*ras*, K-*ras*, N-*ras*, *src*, polyoma MT, *erb B*, *fps*, *mil*, *ros yes*, *sea*) are cytoplasmic [95]. Spandidos and Wilkie [96] were able to transform these cells in a single step with the prototype gene of the second cytoplasmic group, the activated human H-*ras* oncogene, whereas other workers found that this oncogene made the same type of cells metastatic as well [97]. These tumorigenic and metastatic *ras*-transformed rat embryo cells contain numerous chromosomal aberrations. The experience gained with rat embryo cells has served to highlight the potential complexity of this type of system.

An interesting system has been employed by Taparowsky and coworkers [98] using C3H10T1/2 cells, which require multiple oncogenes for the induction of tumorigenesis. Using the same cell line, Herschman and coworkers [99] have found that ultraviolet light can partially transform the C3H10T1/2 cells to form foci that remain sensitive to growth-inhibitory signals from neighboring normal cells.

A valuable system using Syrian hamster embryo (SHE) fibroblasts has been developed by Barrett and coworkers [100]. They found that SHE cells could be transformed to form tumors after the transfection of both v-*ras* and v-*myc* oncogenes. Chromosomal analysis of the resulting *ras*/*myc*-induced SHE tumors revealed the nonrandom loss of chromosome 15. In this system, therefore, three genetic events are required for transformation: *ras* transfection, *myc* transfection, and the loss of chromosome 15. Normal SHE cells cannot be transformed by the *ras* oncogene, but some cell lines immortalized by treatment with diethylstilbesterol (DES) or asbestos are susceptible to *ras* transformation. Somatic cell hybrids of these *ras*-susceptible cells and tumorigenic cell lines remain tumorigenic, but hybrids formed between the parent, *ras*-resistant SHE cells and tumorigenic cell lines give rise to nontumorigenic hybrids. Therefore, the DES- or asbestos-treated cell lines have lost some suppressive function that may be related to the effects of the *myc* oncogene or to the loss of hamster chromosome 15. Newbold and Overall [101] have developed a similar system, also using the

hamster cells. Unfortunately, a complete map of the chromosomal locations of hamster oncogenes is not available.

Human cell systems for studying oncogenes in multistage carcinogenesis have been difficult to establish, because human cells are inherently more resistant to tumorigenic transformation. Sager and coworkers [102] were able to induce a morphological alteration and focus formation by transfecting SV40 viral DNA into human foreskin fibroblasts, but these transfected cells were nontumorigenic in nude mice. However, when the cloned EJ bladder carcinoma H-*ras* oncogene was transfected into the foreskin fibroblasts, morphological transformation or tumorigenicity was not detected. Hurlin and coworkers [103] were able to induce morphological transformation, focus formation, and anchorage independence by transfecting, into diploid human fibroblasts, a plasmid that produces high level expression of an activated human H-*ras* oncogene. However, these cells did not form tumors in nude mice. In sharp contrast, Spandidos and Wilkie [96] were able to induce tumorigenesis in rat embryo fibroblasts with the same plasmid. We have been able to transform a nontumorigenic human osteosarcoma cell line, TE-85, to tumorigenicity by transfection with an activated *ras* oncogene [104].

Human cells apparently possess some impedance factors that render them refractory to transformation; thus, human cell systems are more difficult to employ in studying carcinogenesis. Fusenig and coworkers [105] and R.E. Scott and coworkers [106] have developed interesting systems using human keratinocytes, and have studied the interaction of tumorigenesis and differentiation.

One of the human cell culture systems we developed has allowed us to study the way that an oncogene's action on cells is no longer regulated as cells progress along the transitions in multistage carcinogenesis. The cell line we employ, PA-1 human teratocarcinoma cells, exhibits progression as it is passaged in culture. Initially the cells are weakly tumorigenic, but then they revert in culture, becoming nontumorigenic when injected into nude mice [104]. These cells in culture from passage 30 to 90 are nontumorigenic revertants. As PA-1 cells are further passaged (passage 90 or greater), they grow progressively faster in culture and readily form tumors in nude mice, with a latent growth period of from 7 to 10 weeks [107]. PA-1 cells are highly stable karyotypically, yet they undergo progression in culture after the establishment of the cell line at passage 30. This gives rise, after further growth, to spontaneously transformed tumorigenic cell lines [108, 109].

PA-1 cells at passage 100 and beyond contain an N-*ras* oncogene with an activating point mutation at amino acid position 12 changing from glycine to aspartate [108]. The N-*ras* genes in early passage preneoplastic PA-1 cells do not contain the activating mutation in the first exon of the gene [110]. We used gene transfer studies to determine whether this activated N-*ras* oncogene had a causal role in the tumorigenesis of these cells. We wished to determine whether a nontumorigenic PA-1 cell line could form tumors in athymic nude mice after the activated oncogene was added. For these experiments we used a nontumorigenic clone of PA-1 cells (clone 1) isolated at passage 63. The cloned cells were carried an additional 50 passages (passage 63 + 50) before these experiments, during which time the cells remained nontumorigenic. The activated N-*ras* gene was cloned onto the drug-selectable plasmid pSV2-neo [111] and introduced into clone 1 PA-1 cells. The resulting cells were able to form tumors when injected into nude mice [110]. The introduction of the pSV2-neo vector alone or a pSV2-neo construct containing the normal human N-*ras* proto-oncogene into clone 1 cells did not result in tumor formation in nude mice [112]. Single copy levels of the N-*ras* gene were found in the transfected cells and tumors resulting from their injection into athymic nude mice, indicating that gene amplification of the N-*ras* oncogene sequences is not required for tumor formation by PA-1 clone 1 cells. Tumorigenic transformation of these preneoplastic PA-1 cells could also be achieved by using a pSV2-neo- activated H-*ras* construct. Northern blot analysis of the N-*ras* locus [109] and p21 ras oncogene expression by Western blot analysis indicated that clone 1 PA-1 can be

transformed by normal levels of expression of the oncogene.

Clonal preneoplastic cell lines were established at passage 40 and carried an additional 10 passages; they were than transfected with pSV2-neo oncogene plasmid constructions. When experiments similar to those described above for clone 1 cells were performed on these clone 6 cells (used at passage 40 + 10) using a pSV2-neo N-*ras* or H-*ras* construct, we found that they were not transformed to tumorigenicity in nude mice [109]. We wanted to know whether cells could progress from a preneoplastic stage in which they could suppress the transforming potential of an activated *ras* oncogene to a stage in which they could be transformed by an activated *ras* oncogene: i.e., from Stage 1 to Stage 2. PA-1 clone 1 cells were analyzed at passage 63 + 15 for transformation by the activated N-*ras* oncogene. These cells could not form tumors in nude mice after transfection of the activated N-*ras* oncogene. Since they could be transformed at passage 63 + 15 by the activated N-*ras* oncogene, we concluded that PA-1 cells can progress during 35 passages in culture from a stage in which they suppress *ras* to one in which they can be transformed by *ras*. Another *ras*-resistant stage 1 cell, clone 6, also progressed to a cell that displayed the *ras* transformable phenotype within 25 passages in culture. Introduction of either the PA-1 N-*ras* or T24 H-*ras* into Stage 1 cells at passage 40 + 15 did not induce neoplastic transformation. However, when the T24 H-*ras* was introduced into passage 40 + 40 clone 6 cells or 63 + 50 clone 1 cells they formed colonies in soft agar and tumors in nude mice. We thus have two examples of clonal cell lines that can progress from *ras* resistant to *ras* transformable by passage in culture.

We can now identify multiple stages of cell progression toward tumorigenesis: stage 1 (*ras*-resistant), stage 2 (*ras*-transformable), stage 3 (*ras*-transformed PA-1 cells: GT, induced by gene transfer on an activated N-*ras* oncogene, or SP, containing a spontaneously activated N-*ras* oncogene), and stage 4 (transformed cells that have been selected through intrasplenic growth in nude mice to form liver metastases) [104]. Stage 1 PA-1 cells cannot be transformed to anchorage-independent growth or to form tumors in nude mice by a *ras* oncogene alone. However, they can be transformed by a plasmid containing an activated H-*ras* oncogene [108] and v-*myc* [113] oncogene cloned together into pSV2-neo [109]. In addition, because the level of endogenous N-*ras* expression does not vary among the four stages of PA-1 cell progression [114], we believe that the control of oncogene-inducible transformation is not regulated at the level of oncogene RNA expression (Table 4).

A central question is the nature of the mechanism by which one cell can progress from *ras*-resistant (stage 1 cells) to *ras*-susceptible (stage 2 cells). Because v-*myc* could cooperate with *ras* to transform stage 1 cells, as was observed by Weinberg and coworkers [22] for rat embryo fibroblasts, we investigated whether it was due to the overexpression of a nuclear oncogene. We found no differences in nuclear oncogene expression (Table 4)

We could not account for the transformability of stage 2 cells by the expression of a cooperating oncogene whose expression was absent from stage 1 cells. An alternative hypothesis was that stage 1 cells contained a suppressor function that was lost in stage 2 cells. To test this hypothesis, we formed somatic cell hybrids between stage 1 and stage 2 cells in the presence of an activated N-*ras* oncogene (*ras*-transformed stage 3 cells), and we found that these hybrids were nontumorigenic. The loss of specific chromosomes after passaging in culture allowed those hybrids to become tumorigenic [115]. We have found that in somatic cell hybridization experiments, cells possessing tumor-suppressing activity cannot be transformed by *ras*. These cells also cannot be stimulated by multiple growth factors. Only preneoplastic PA-1 cells that are transformable by activated *ras* oncogenes (stage 2) can be stimulated by epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF), and transforming growth factor alpha (TGF-alpha) to exhibit anchorage-independent growth; *ras*-resistant cells (stage 1) do not grow in agar with these growth factors. We are now investigating the ability of EGF, b-FGF, or TGF-alpha to affect growth factor-responsive gene ex-

pression in Stage 1 versus Stage 2 PA-1 cells in order to understand how these mechanisms interact.

In summary, certain preneoplastic cells in this PA-1 series are susceptible to transformation by *ras* oncogenes, while others have not progressed as far and require a *myc* oncogene in addition to the *ras* oncogene to induce tumorigenesis. The molecular genetic basis of the susceptibility to single oncogene-induced transformation appears to be due to the loss of a tumor suppressor gene. The *myc* oncogene must therefore bypass the regulatory effects of the suppressor gene. We have found that the mechanism by which cells acquire the susceptibility to tumorigenic transformation by an activated *ras* oncogene is related to responsiveness to growth factors found in many normal tissues (EGF, TGF-alpha, and b-FGF), possibly by affecting an autocrine mechanism. If the cells become responsive to these growth factors and the oncogene induces secretion of the growth factor, then the cell can stimulate its own growth. This loss of regulation of growth factor-mediated stimulation of growth represents a new regulatory role for tumor suppressor genes during preneoplastic stages progressing toward tumorigenicity.

Properties of fibroblasts from patients with inherited cancers

In order to study the causative role genes play in the induction of tumors, we sought an experimental system in which cells occur naturally in an abnormal but preneoplastic stage. Normal cells from individuals with an inherited predisposition to cancer might possess some of the genetic changes that make cells susceptible to oncogene-induced transformation. For these studies we used dermal fibroblasts from patients in families with soft tissue sarcomas, a syndrome originally described by Li and Fraumeni [116, 117]. Families with the Li-Fraumeni syndrome show an inherited pattern of sarcomas and various other types of cancers that follow a dominant mode of transmission, have an early age at onset, and exhibit multiple primary tumors. Approximately 7% of families with childhood soft tissue sarcomas fit a model for the autosomal dominant mode of transmission of cancer in their families. As fibrosarcomas are frequently observed in this syndrome [118, 119], the fibroblasts offered an 'at risk' target tissue for observing phenotypic characteristics of this unusual syndrome.

One hypothesis we wanted to test was whether the normal cells from Li-Fraumeni patients existed in a more progressed stage of the carcinogenic progress than the same types of cells from normal

Table 4. Summary of Northern blot analysis of PA-1 cells

Cell stage					
Stage genes	1	2	3GT	3SP	4
c-myc	–	–	–	–	–
N-myc	–	–	–	–	–
c-fos	–	–	–	–	–
c-myb	+++	+++	+++	+++	+++
p53	+++	+++	+++	+++	+++
c-jun B	+	+	+	+	+
c-N-ras	+++	+++	+++	+++	+++
c-H-ras	+	+	+	+	+
c-K-ras	++	++	++	++	++

+ = 14-day exposure of autoradiograph to produce a dark band.
++ = 7-day exposure of autoradiograph to produce a dark band.
+++ = 2-day exposure of autoradiograph to produce a dark band.

donors. We investigated the *in vitro* growth characteristics of fibroblasts derived from skin biopsies of patients with Li-Fraumeni syndrome and unaffected controls. Fibroblasts from control donors maintain a normal morphology and had a short and finite lifespan in culture. Fibroblasts from seven out of eight affected individuals from Li-Fraumeni families develop changes in morphology and chromosomal abnormalities, and enter a growth crisis during which they begin to senesce (in a fashion similar to fibroblasts from normal donors), but then they recover. The cells then grow rapidly and maintain the morphology of a transformed cell. The chromosomal abnormalities are significant in that even prior to their emergence from senescence, essentially all the metaphases examined contained abnormal numbers, numerous damaged chromosomes, and evidence for gene amplification in the form of double minute chromosomes and homogeneously staining regions. The spontaneous *in vitro* transformation of normal human fibroblasts is a rare event [120]. Hayflick had shown that normal human diploid fibroblasts have a limited lifespan when grown *in vitro;* they cease to divide when subcultured at confluency after approximately 30–50 population doublings (pd) [121]. Thus, the spontaneous acquisition of an infinite lifespan in culture of the skin fibroblasts with the associated aneuploidy and anchorage independent growth, extremely rare events in cells derived from normal donors, occurs frequently in cells derived from Li-Fraumeni patients and may have predictive value in screening for gene carriers at high risk of cancer.

Although the Li-Fraumeni fibroblasts have a prolonged lifespan in culture (in one case more than 300 population doublings in culture) and exhibit anchorage-independent growth, they are nontumorigenic in athymic nude mice. We decided to test whether these immortalized fibroblasts had progressed in culture to be susceptible to *ras* oncogene-inducible transformation. We were able to induce tumorigenic transformation of these fibroblasts by transfection of an activated H-*ras* oncogene. The *ras* oncogene-bearing fibroblasts formed tumors in nude mice with a 3-week latent period, which is remarkably rapid for human cells. Reinjection of cells derived from these tumors also formed tumors in nude mice with 3-week latent period, indicating that no further genetic changes were necessary for the initial transformation to occur. An activated v-*myc* oncogene was unable to transform these cells to form tumors. These immortalized fibroblasts undergo many steps associated with carcinogenesis and provide a useful model for studying the role of oncogenes, tumor suppressor genes, and cancer susceptibility genes in human tumorigenesis.

Table 5. Tumorigenicity of transfected PA-1 cells

Cell	Plasmid	Tumors incidence	Latency (wks)	Stage
Clone 6 cells				
P40 + 15	neo	0/3	>36	1
	neo + N-*ras*	0/3	>36	
p40 + 15	neo	0/3	>36	1
	neo + T24/H-*ras*	0/3	>36	
P40 + 40	neo	0/3	>36	2
	neo + T24/H-*ras*	3/3	9	
Clone 1 cells				
P63 + 15	neo	0/3	>36	1
	neo + N-*ras*	0/3	>36	
P63 − 50	neo	0/3	>36	2
	neo + N-*ras*	2/3	8,13	
P63 + 50	neo	0/3	>36	2
	neo + T24/H-*ras*	2/3		

Conclusions

Human cell systems provide advantages over rodent models in that (1) they are not inbred and genetically homozygous at all genetic loci; (2) we have isolated clonal pseudodiploid cells that represent presumptive stages between normal cells and malignant tumors; (3) we can study differentiation and carcinogenesis in a single model system; and (4) many of the genetic elements, i.e., oncogenes and tumor suppressor genes deemed important in other systems, have been involved in the progression of PA-1 cells toward tumorigenicity. We have developed a model for spontaneous immortalization of human fibroblasts, and we have shown that immortalization is required for *ras* transformability.

Key unanswered questions

While human cell culture systems have allowed us to begin to answer questions on how oncogenes and suppressor genes interact, we have only begun to address these processes mechanistically. We have to be able to identify how *myc* can abrogate the effects of the tumor suppressor. We also want to identify how the suppressor controls both signal transduction and *ras* transformability. Using fibroblasts from patients with inherited cancers we expect to use the properties we have identified and new genetic markers we are cloning that are specific for immortalized cells to develop a short-term assay to detect genetic predisposition to cancer.

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