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INTRODUCTION OF CHROMOSOME 15 INTO THE IMMORTALIZED HUMAN FIBROBLAST CELL STRAIN MSU-1.1 PREVENTS MALIGNANT TRANSFORMATION BY THE T24 H-RAS ONCOGENE

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INTRODUCTION OF CHROMOSOME 15 INTO THE IMMORTALIZED HUMAN FIBROBLAST CELL STRAIN MSU-1.1 PREVENTS MALIGNANT TRANSFORMATION BY THE T24 *H-RAS* ONCOGENE

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ABSTRACT

INTRODUCTION OF CHROMOSOME 15 INTO THE IMMORTALIZED HUMAN FIBROBLAST CELL STRAIN MSU-1.1 PREVENTS MALIGNANT TRANSFORMATION BY THE T24 *H-RAS* ONCOGENE

By

Evan Louis Kaplan

Although diploid mortal and immortal human skin fibroblasts cannot be transformed into malignant cells by T24 *H-RAS* oncogene overexpression, a cell strain derived from diploid immortal human fibroblasts (MSU-1.1) was readily transformable into malignant cells by overexpression of the T24 *H-RAS* oncogene (Hurlin *et al.*, 1989). MSU-1.1 cells differ from diploid mortal and immortal human fibroblasts in that they exhibit a loss of DNA fragments on chromosomes 11 (11p15→11pter), 12 (12q11.2→12pter), and 15 (15p11.2→15pter) and possess a partial trisomy of chromosome 1 (1qter→1p13). These genetic alterations suggest that one or more of the lost DNA fragments may be required for the malignant transformation of these cells by the T24 *H-RAS* oncogene. To test this hypothesis, we transferred a normal human 11, 12, or 15 chromosome into an MSU-1.1 cell strain (MSU-1.1 TG^R). The resultant chromosome hybrid clonal populations were assessed for malignant transformability by transfection with the T24 *H-RAS* oncogene. The results indicate that the introduction of chromosome 15, but not chromosomes 11 or 12, prevented the malignant transformation by T24 *H-RAS* oncogene

overexpression. The T24 *H-RAS* transformed MSU-1.1 chromosome 15 hybrid clonal populations had *in vitro* properties similar to those of the control T24 *H-RAS* transformed MSU-1.1 cell strain; both exhibited a transformed morphology, similar growth rates, and the ability to form colonies in agarose. However, the control T24 *H-RAS* transformed MSU-1.1 cell strain, the hybrid clonal populations did not form tumors when injected into athymic mice. The introduction of chromosome 11 or 12 had no effect on T24 *H-RAS* transformation of MSU-1.1 cells into malignant cells. To verify these findings, we transferred a normal human 11, 12, or 15 chromosome into an MSU-1.1 T24 *H-RAS* transformed tumor-derived cell strain, PH2MT, which is karyotypically identical to the MSU-1.1 cell strain. Just as with the MSU-1.1 cells, the introduction of chromosome 15 into PH2MT cells resulted in clonal populations that were not tumorigenic or were only slightly tumorigenic. In addition, a majority of the non-tumorigenic hybrid clonal populations lost the characteristics of the T24 *H-RAS* transformed cells. These cells now had a spindle-shaped morphology, grew at a slower rate, and did not form colonies in agarose. The introduction of chromosome 11 or 12 into the PH2MT cell strain had no effect on morphology, growth rate, ability to form colonies in agarose, or tumorigenicity of the resultant hybrid clonal populations. These novel findings suggest tumor suppressor activity localized to chromosome 15. This is the first demonstration that the introduction of chromosome 15 into either immortal or tumorigenic cell strains can suppress or reverse the tumorigenicity state of the recipient cell strain.

DEDICATION

This dissertation is dedicated to My Love.

Ahhh Barn; He is all things good.

I would also like to dedicate this dissertation to my parents, parent in-laws, S and S, brothers, brother in-laws, sister in-laws, and friends for their love and moral support.

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LIST OF ABBREVIATIONS

ACF	aberrant crypt foci
ALT	alternative lengthening of telomeres
APC	adenomatous polyposis coli gene
ATM	ataxia telangiectasia mutated
bHLHZ	basic helix-loop-helix-zipper
CAM(s)	cellular adhesion molecule(s)
Cdk(s)	cyclin-dependent kinase(s)
DCC	deleted in colorectal carcinoma gene
DNA-PK	DNA-dependent protein kinase
DSB(s)	DNA double-strand break(s)
DPC4	deleted in pancreatic cancer 4 gene
E2F	E2F family of transcription factors
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal regulated kinase
FAP	familial adenomatous polyposis
FBS	fetal bovine serum
FISH	fluorescent *in situ* hybridization
GADD45	growth arrest DNA damage 45 gene

GTP

GDP

HNP

HPV

hTE

L-F

LO

MA

M

M

M

N

Y

I

GTP	guanosine triphosphate
GDP	guanosine diphosphate
HNPCC	hereditary nonpolyposis colorectal cancer
HPV	human papilloma virus
hTERT	human telomerase reverse transcriptase gene
L-F	Li-Fraumeni syndrome
LOH	loss of heterozygosity
MAPKKK	Mitogen activated protein kinase kinase kinase
MDM2	Mouse double minute 2 gene
MEK	MAP/ERK kinase
MEKK	MAP/ERK kinase kinase
MEM	modified Eagle's medium
MMCT	microcell mediated chromosome transfer
MMP(s)	matrix metalloproteinase(s)
NER	nucleotide excision repair
NHEJ	nonhomologous end-joining
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PEG	polyethylene glycol
PHA-P	phytohemaglutinin-P
PI3-kinase	phosphatidylinositol 3-kinase
pRb	retinoblastoma protein

RB

SCS

SOS

SH2

SH3

TBS

TG

TF

TG

TC

TI

TS

V

X

RB	retinoblastoma gene
SCS	supplemented calf serum
SOS	son of sevenless
SH2	Src homology 2 binding domain
SH3	Src homology 3 binding domain
TBST	Tris-buffered saline with tween
TG^R	thioguanine-resistant
TFO	triple helix-forming oligonucleotides
TGF-α	transforming growth factor-α
TGF-β	transforming growth factor-β
THBS1	thrombospondin-1 gene
TSG(s)	tumor suppressor gene(s)
VEGF	vascular endothelial growth factor
XP	xeroderma pigmentosum

INTRODUCTION

It is estimated that in the year 2001, 1,268,000 new cases of cancer will be diagnosed in the Unites States and an estimated 553,400 individuals will die from the disease (1). Cancer is the result of genetic alterations caused by spontaneous mutations, mutations induced by environmental factors (carcinogens), or inherited genetic mutations (2). In addition, epigenetic alterations that change gene expression, may also play a role (3, 4). These alterations occur over time in a multi-step process, with many of the changes, perhaps all, conferring on a cell a selective advantage that allows it to replicate and form a large number of cells (e.g. a million) with that particular alteration. This clonal expansion greatly increases the probability that a second cancer-related alteration (mutation) will occur in a cell that already contains an initial alteration. Given sufficient time, this multi-step process might result in a cell that has acquired all of the functions necessary for it to give rise to a cancer (3, 4). These functions include growth stimulatory signal autonomy, insensitivity to growth inhibitory signals, infinite proliferative capacity (cellular immortalization), angiogenic potential (the ability to form new blood vessels and capillaries), and tissue invasion and metastasis.

Neoplasms, commonly called tumors, are defined as heritably altered, relatively autonomous growths of tissue (5). Neoplasms can be either malignant or benign. Benign neoplasms lack the ability to invade the surrounding tissue or

form secondary neoplasms at locations away from the primary site (a process termed metastasis). Examples of benign neoplasms are fibromas derived from fibrous connective tissue, lipomas derived from lipoblasts, and adenomas derived from glandular cells. In comparison to benign neoplasms, malignant neoplasms have the ability to invade the surrounding normal tissue and metastasize. The name "cancer" refers solely to malignant neoplasms. Malignant neoplasms are the most dangerous to patients because they invade and compromise essential vital organs. There are approximately two hundred distinct varieties of human malignant neoplasms, which can be categorized into two main classes (6). Ninety percent of malignant neoplasms are carcinomas, which originate from epithelial tissues (skin, the linings of the respiratory and digestive tracts, and glandular tissue). Examples of carcinomas are lung cancer, breast cancer, and intestinal cancer. The remaining ten percent of malignant cancers are sarcomas, which originate from the tissues of the body derived from the mesoderm. These tissues include bone, cartilage, muscle, fibrous, and fat tissue. Examples of sarcomas are fibrosarcomas, osteosarcomas and liposarcomas. Because of their unique characteristics, leukemias are generally treated as a separate class, although they technically are cancers arising from mesenchymal tissues. These tumors originate from the blood-forming tissues, such as bone marrow, and are characterized by the overproduction of specific types of immature blood cells. Leukemias are further classified by the rate of progression, rapid (acute) or slow (chronic).

As noted above, cancer is the result of a multi-step process that involves several genetic alterations, and it is the accumulation of these alterations over time that results in tumor formation. To better understand this multi-step process, various human cell types have been studied *in vitro* (7-11). One such *in vitro* multi-step model is the MSU-1 lineage of cells (11-14). The MSU-1 lineage of cells represents a series of cell strains, each derived from its predecessor by clonal selection, and contains transformed immortal non-malignant and malignant cell strains. This lineage began with a fibroblast cell line obtained from the foreskin of a neonate, designated LG1. The first transformed immortal cell strain in the lineage, designated MSU-1.0, is diploid, karyotypically stable (11), and telomerase positive (15). A spontaneous derivative of MSU-1.0, designated MSU-1.1, is near-diploid and karyotypically stable (11). Both MSU-1.0 and MSU-1.1 cells are non-malignant. The MSU-1.1 cell strain differs from the MSU-1.0 cell strain in that it exhibits a growth advantage in culture and contains 45 chromosomes, including two marker chromosomes (11, 16). Marker M1 is made up of a translocation between a partial trisomy of chromosome 1 (1qter→1p13) and most of chromosome 11. The missing region of chromosome 11 on this marker is the region 11p15 to the p arm terminus; t(1;11)(1qter→1p13 : 11p15→11qter). Marker M2 is made up of a translocation between chromosome 12 and chromosome 15, in which the entire p arm and a small portion of the q arm of chromosome 12 and approximately half of the p arm of chromosome 15 have been lost; t(12;15)(12qter→12q11.2 : 15p11.2→15qter). Thus, there are

four genetic alterations involved in the formation of the two marker chromosomes: losses of chromosomal DNA on chromosome 11 (11p15→11pter), chromosome 12 (12q11.2→12pter), and chromosome 15 (15p11.2→15pter), as well as a gain of chromosomal DNA because of the partial trisomy of chromosome 1 (1qter→1p13).

Neither LG1, the diploid finite life-span fibroblastic cell line from which the MSU-1 lineage arose, nor the infinite life-span diploid derivative, MSU-1.0 cell strain, has ever been successfully transformed into malignant cells by overexpression of the T24 *H-RAS* oncogene (17, 18). Furthermore there have been no reports on the successful malignant transformation of other human diploid finite or diploid infinite life-span fibroblast cell strains. However, the near-diploid infinite life-span fibroblastic cell strain MSU-1.1, a spontaneous derivative of the diploid infinite life-span cell strain MSU-1.0, can be malignantly transformed by overexpression of the T24 *H-RAS* oncogene (13). Based on the demonstration that MSU-1.1 cells can be malignantly transformed by overexpression of the T24 *H-RAS* oncogene but MSU-1.0 cells cannot, I hypothesize that the loss of chromosomal material located on chromosomes 11, 12, or 15, resulting in the formation of one or the other (or both) marker chromosomes found in the MSU-1.1 cell strain, allows MSU-1.1 cells to be malignantly transformed by overexpression of the T24 *H-RAS* oncogene.

This dissertation consists of two chapters. Chapter I is a review of the literature providing background on the biological and molecular basis of cancer formation and development. It includes detailed descriptions of the multi-step process of carcinogenesis and the genetic alterations involved. Since cancer results from the accumulation of specific genetic alterations, well-characterized examples of genes that are commonly altered in the cancer process are described in detail. In addition, a detailed description of acquired abilities that are necessary for normal cells to transform to cancer cells is provided. Chapter I concludes with a discussion of two models of the multi-step process of carcinogenesis. The first model, the colorectal cancer model, has provided *in vivo* evidence how specific genetic alterations correlate with specific stages of tumor development and progression. Detailed descriptions of the forms of colorectal cancer and the genetic alterations involved are discussed. The second model is the MSU-1 *in vitro* model. Detailed descriptions of the MSU-1.0 and MSU-1.1 cell strains are provided. In addition, a characterization of each of the lost chromosomal regions and the partial trisomy of chromosome 1 is discussed.

Chapter II is a manuscript prepared for submission to the journal *Cancer Research.* In order to test my aforementioned hypothesis, chromosomes 11, 12, and 15 were individually introduced by microcell mediated chromosome transfer (MMCT) into MSU-1.1 cells. The results obtained from this study indicate that

chromosome 15, but not chromosome 11 or 12, inhibited malignant transformation of the MSU-1.1 chromosome hybrid clonal populations by overexpression of the T24 *H-RAS*. In support of these findings, introduction of chromosome 15, but not chromosome 11 or 12, into the MSU-1.1 T24 *H-RAS* transformed tumor-derived cell strain, PH2MT, suppressed tumorigenicity of the PH2MT chromosome hybrid clonal populations.

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CHAPTER 1

LITERATURE REVIEW

I. The Multi-step Process of Carcinogenesis

Carcinogenesis is a multi-step process in which alterations in gene function cause a normal cell to become a cancer cell by acquiring several new properties, including growth stimulatory signal autonomy, insensitivity to growth inhibitory signals, infinite proliferative capacity, angiogenic potential, and tissue invasion and metastasis. Genes in which a loss of function plays a causal role in cancer are referred to as tumor suppressor genes (TSGs) (1). Genes which exhibit a gain of function in cancer are referred to as oncogenes (1). The normal functioning form of an oncogene is known as a proto-oncogene. TSGs and oncogenes are generally involved in regulating cell growth and proliferation, but some are involved in other specific functions in cancer development, such as angiogenesis and metastasis. Gene function can be altered in many ways, including DNA damage and epigenetic modifications.

DNA damage can be produced by a variety of chemical and physical agents. Chemical carcinogens such as benzo[a]pyrene (found in cigarette smoke and exhaust fumes) and dimethylnitrosamine (formerly used as a chemical solvent) can produce DNA adducts, which can result in DNA frameshifts and mismatch base-pairing (2-6). Other DNA damaging agents include ultraviolet and ionizing radiation. UV radiation commonly produces

covalent linking between adjacent pyrimidine nucleotides resulting in pyrimidine dimers (7). Ionizing radiation produces DNA single- and double-strand breaks, which can result in chromosomal deletions and rearrangements (8, 9). DNA damage can also occur spontaneously because of errors in DNA replication (10). DNA damage is usually repaired. However, if the damaged DNA is not repaired before DNA replication occurs, DNA polymerases may insert an incorrect base opposite the damage (mismatch base-pairing). Subsequent DNA replication will introduce the corresponding incorrect nucleotide into the daughter DNA strand, creating a mutation.

There are several genes involved in DNA damage recognition and cellular repair mechanisms (7). Two examples of DNA damage recognition genes are the gene for the protein kinase, ATM (ataxia telangiectasia mutated) (11, 12), and the gene for BRCA1, often found mutated in breast cancer (13, 14). ATM recognizes DNA double-strand breaks and initiates the repair process by phosphorylating necessary repair proteins, such as BRCA1 (11-14). Mutations to genes involved in cellular repair mechanisms, such as the nucleotide excision repair (NER) genes, can result in several rare recessive photosensitive syndromes (15, 16). For example, people suffering from xeroderma pigmentosum (XP) are predisposed to sunlight-induced skin cancers such as melanomas and basal cell and squamous cell carcinomas. This syndrome is caused by mutations in NER genes, resulting in the expression of non-functional proteins that are unable to repair DNA damage caused by ultraviolet radiation

(15, 16). Cell fusion experiments between normal cells and cells from XP patients have led to the identification of seven gene complementation groups (*XP-A* to *XP-G*) involved in nucleotide excision repair (7). In addition, there is a distinct group of XP patients that have normal DNA repair. These individuals are designated XP variants and this form of XP is the result of mutations to the *XP-V* gene (17). The *XP-V* gene encodes a DNA polymerase involved in error-free replication past UV-damaged nucleotides (18, 19). Loss of function of DNA damage recognition and repair genes (like *ATM*, *BRCA1*, and *XP-A* to *XP-G*) results in an increased mutation frequency and thus, an increase in the probability of TSG loss of function or oncogene gain of function.

Mutations alter gene function by affecting gene expression or activation. However, gene function can also be altered via epigenetic DNA modifications such as methylation or acetylation (20, 21). These modifications can result in the inhibition of gene expression by causing structural changes within the DNA or by blocking the direct binding of transcription factors to the promoter regions of genes (discussed in greater detail below). Such DNA modifications can result in heritable changes in gene expression, but without direct modification of the DNA sequence (22).

As noted above, carcinogenesis is a multi-step process in which biochemical events result in altered gene function, that results in the acquisition of one or more properties involved in the carcinogenesis process. This process involves an initial alteration, such as the loss of function of a TSG or gain of

function of a proto-oncogene. Such changes typically result in the cell having a growth advantage over the surrounding normal cells. Clonal expansion of the altered cell increases its chance of acquiring an additional alteration in a TSG or proto-oncogene, such that upon the n^{th} alteration, the cell is now able to form a tumor and invade adjacent tissue (Figure 1). Because the probability of all these genetic and epigenetic alterations occurring simultaneously in a single cell is infinitesimally small, the process of clonal expansion is very important in each step of carcinogenesis (23-25). After each alteration, clonal expansion is repeated increasing the probability that a cell that has already acquired a particular genetic change related to cancer is susceptible to another alteration. As subsequent genetic and epigenetic alterations are amassed, the cells further progresses towards malignancy. The number of alterations and the specific genes involved in this step-wise process from normal cell to malignant cell are unknown for most cancer types, but epidemiological (26) and *in vitro* (27, 28) studies suggest the number is less than ten.

There are two well-characterized examples of the multi-step process of carcinogenesis. The first is the classical *in vivo* example of the formation of human colorectal tumors. In such tumors, there are alterations involving genetic mutations, chromosomal amplifications, deletions and translocations, and DNA methylation (27, 29-33). A second example is an *in vitro* model involving the

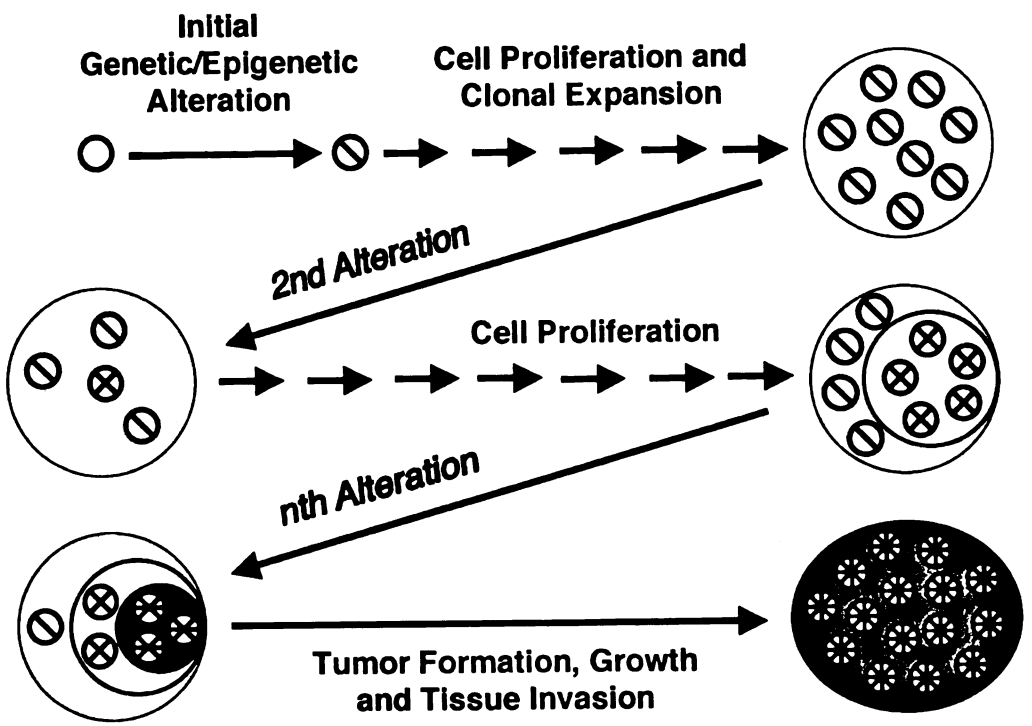

Figure 1. The multi-step process of carcinogenesis. Adapted from (34).

MSU-1 lineage of cells (28, 35). The MSU-1 lineage of cells represents a series of cell strains, each derived from its predecessor, following this multi-step process. This lineage is derived from normal human foreskin fibroblasts and contains immortal non-malignant and immortal malignant cell strains (36-39). Both models are discussed in detail in Section IV.

II. Classes of Genes Critically Involved in Carcinogenesis

As noted above, carcinogenesis is a multi-step process that involves altered gene expression and activation caused by DNA mutations or epigenetic modifications. These alterations affect the functions of genes, and in cancer, often result in the loss of function of TSGs and the gain of function of proto-oncogenes.

A. Tumor Suppressor Genes

The first evidence for the presence of genes in normal cells that suppressed tumorigenicity arose from studies in which cell fusion experiments were performed between malignant and normal diploid mouse cell lines (40, 41). The resultant hybrid cells that had retained the complete chromosome sets of both parental cells were non-malignant as measured by tumor formation in irradiated, newborn mice. These studies suggested that there were critical gene(s) present in the normal cells that suppressed tumor formation of the hybrid cells, and that malignancy results from cells that have lost the function of critical gene(s) that must be functional/active to prevent tumor formation.

The hypothesis that loss of gene function is involved in tumor formation was further supported by the statistical findings of Knudson in his research involving the childhood cancer, retinoblastoma (42-44) (the retinoblastoma cancer and gene are discussed in greater detail below). Based upon population incidence curves, Knudson hypothesized that two rate-limiting genetic events

were sufficient to cause retinoblastoma (Knudson's "two-hit" hypothesis). These rate-limiting events resulted in the independent inactivation of both homozygous (identical) alleles of a "retinoblastoma" gene, later identified and designated *RB* (64-66). The first mutation occurs in one allele of the gene in a germinal (inherited) cell or somatic (non-inherited) cell resulting in heterozygosity of that gene (i.e. one normal allele and one mutated allele of that gene), and a second somatic mutation occurs in the other allele of the gene resulting in a cell capable of forming a retinoblastoma.

It is now recognized that the second mutation assumed by Knudson's "two-hit" hypothesis results in loss of heterozygosity (LOH), which produces hemi- or homozygosity of the gene of interest (45, 46). The acquisition of a mutation in the second copy of the gene (resulting in LOH) can occur by various mechanisms, including DNA deletion, unbalanced translocation, mitotic recombination, and mitotic non-disjunction with re-duplication of the chromosome carrying the mutated TSG (45, 46). LOH has been detected in various TSGs involved in human primary breast carcinoma (47), colorectal cancer (48), liver carcinoma (49), as well as many other cancers (50). Although LOH can be demonstrated for most tumors in which TSGs play a role, it has not been demonstrated in every case. Other mechanisms, such as epigenetic modifications and haplo-insufficiency, can also play a role in TSG loss of function.

Epigenetic DNA modifications can also contribute to heritable changes in gene expression without direct modification of the DNA sequence (22). The most common epigenetic modification is methylation of DNA, but other epigenetic mechanisms exist (51). DNA methylation involves DNA methyltransferases that catalyze the post-replicative addition of a methyl group (CH_3) to nucleotides. The cofactor S-adenosylmethionine is the universal donor for this process (52). Methylation generally occurs at the C5 position of cytosine resulting in the "fifth" base, 5-methylcytosine (53). Frequent targets of methylation are CpG islands located in promoter regions of genes (53). CpG islands are GpC- and CpG-rich regions between 200 base pairs to several kilobases in length. Hypermethylation of gene promoter regions can prevent gene expression by blocking the direct binding of transcription factors or by causing structural changes in the gene (29, 53). DNA hypermethylation has been shown to play a role in the silencing of several genes in cancer involved in DNA repair, apoptosis, cell cycle regulation, signal transduction, and transcription (53-55). It has been recently suggested that the "two-hit" theory should also include epigenetic modifications, such as DNA methylation (Figure 2) (20, 21, 55).

In addition to LOH and epigenetic modifications, haplo-insufficiency might also be involved in loss of gene function (56). Haplo-insufficiency can occur when one allele of a gene is inactivated by a mutation and the other allele does not produce a sufficient amount of protein product required for normal function. Haplo-insufficiency has been detected for the *PTEN* gene, which encodes a lipid

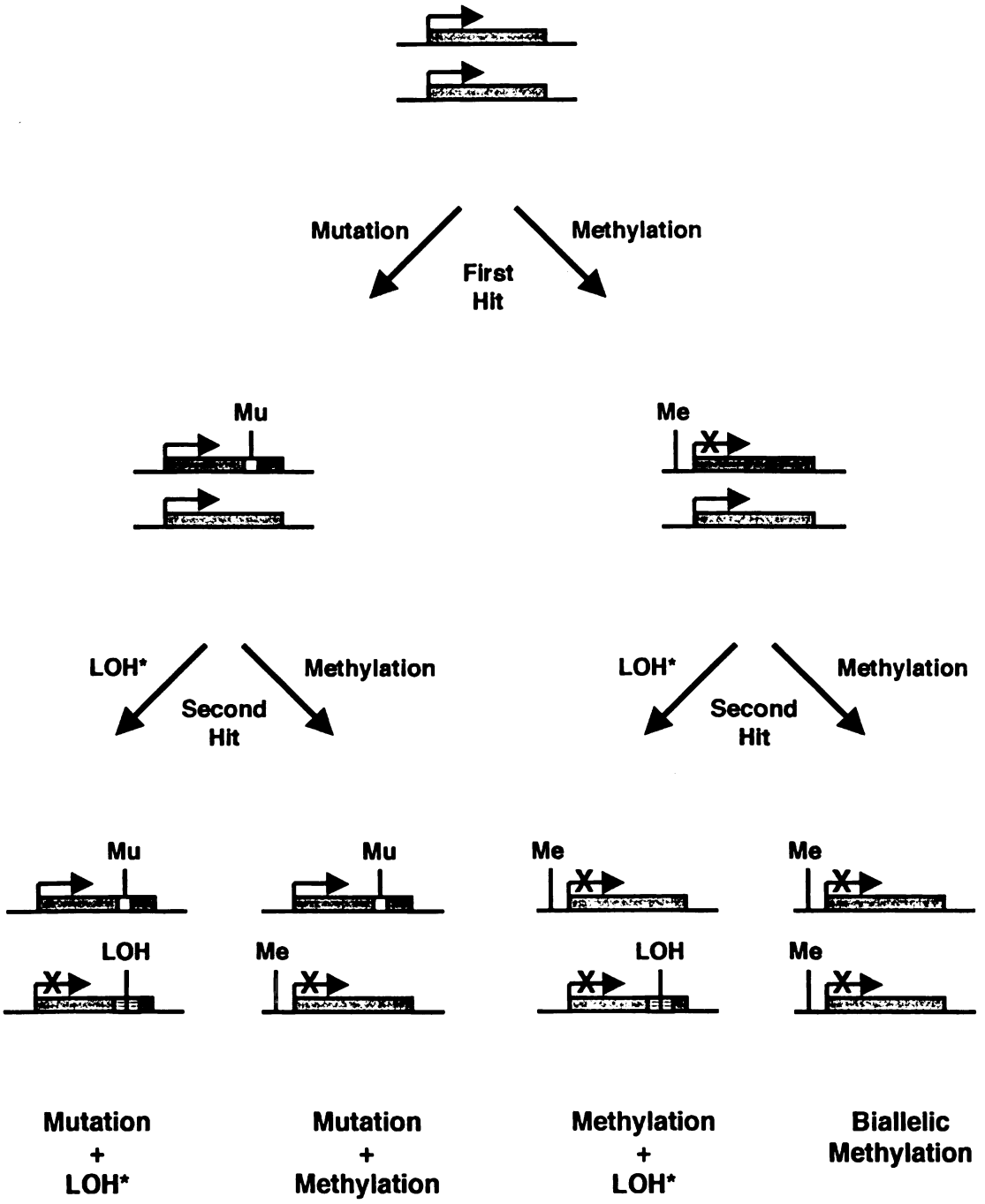

Figure 2. Knudson's "two-hit" hypothesis revised to include methylation. Adapted from (55). LOH* can occur by DNA deletion, unbalanced translocation, mitotic recombination, and mitotic non-disjunction with re-duplication of the chromosome carrying the mutated TSG (45, 46).

phosphatase that negatively regulates the phosphatidylinositol 3-kinase (PI3-kinase) pathway (57), the *p27^{Kip1}* gene, which is involved in inhibiting Cdks (cyclin-dependent kinases) and cell proliferation (58), and various other TSGs (56). Like epigenetic modifications, it has also been suggested that the "two-hit" theory should include haplo-insufficiency as one mechanism for the second "hit" (59).

There are approximately thirty known TSGs that have been cloned and characterized that are involved in various cancer types (60). TSG actions are generally mediated through signal transduction pathways that either negatively regulate cell proliferation or promote programmed cell death (apoptosis). The *p53* and *RB* genes are classical TSGs and are discussed in greater detail below. Some TSGs are involved in other aspects of carcinogenesis not directly associated with cell cycle regulation. Two examples are the *ST7* gene (61) and the thrombospondin-1 gene, *THBS1*, an inhibitor of angiogenesis (62) (discussed in greater detail below). *ST7* is a novel TSG discovered in the Carcinogenesis Laboratory at Michigan State University, that has decreased expression in various human tumor derived cell strains and in *in vitro* malignantly transformed cell strains relative to normal human fibroblasts (61). *ST7* is a member of the family of low density lipoprotein receptor-related proteins (61), which are cell surface endocytic receptors, involved in binding to and internalizing extracellular ligands for degradation by lysosomes (63). The exact role that *ST7* plays in the

carcinogenesis process and the ligands that bind to this receptor are currently being investigated.

TSGs are recessive-acting; that is, one normal functioning allele normally produces sufficient expression of protein for cell function (the exception being in those cases where haplo-insufficiency occurs). Thus, for most TSGs, both alleles must lose the ability to express functional protein for loss of cell function to occur. The two "classical" TSGs are *RB/p105*, the first TSG identified as a critical gene involved in carcinogenesis (64-66), and *p53*, the most frequently mutated gene involved in carcinogenesis (67, 68).

1. The Retinoblastoma Tumor Suppressor Gene

The retinoblastoma gene (*RB/p105*) was initially discovered to be mutated in retinoblastoma, a rare form of cancer that originates from retinal sensory cells and occurs in early childhood (69). *RB/p105* has since been found to be mutated in a variety of other cancer types (70). Besides *RB/p105*, there are two additional *RB* family members, *p107*, and *RB2/p130*, both of which have similar function to *RB/p105* (71-73).

a. Function

The *RB/p105* protein (pRb) is a phosphoprotein involved in cell cycle regulation and apoptosis (70, 74). pRb regulates the cell cycle at the G_1 to S phase transition by binding to members of the E2F family of transcription factors

(E2F) (73). DNA binding sites for E2F are located in the promoter region of many genes involved in cell cycle progression, such as cyclin A, cyclin E, *Cdc2*, and *Cdk2* (73). In G_0 and early G_1, phosphate groups are removed from pRb by PP1 (phosphoprotein phosphatase 1) (75). The hypophosphorylated form of pRb binds to E2F, repressing E2F-mediated transcription (73). During late G_1 and early S phase, pRb is phosphorylated by the cyclin D-Cdk4/6 complexes (70, 76). Phosphorylation of pRb causes a conformational change resulting in the release of E2F, which then participates in a transcriptional complex with RNA polymerase to induce expression of genes required for cell cycle progression into and through S phase. In mid-late S phase, E2F is phosphorylated by the cyclin A/Cdk2 complex, resulting in inactivation of E2F (77). In addition to cell cycle regulation, pRb is also involved in apoptosis initiation via E2F release (78). Unbound E2F activates $p14^{ARF}$, resulting in the inhibition of MDM2 (mouse double minute 2) mediated degradation of p53 (discussed in detail below), which can lead to the initiation of apoptosis.

b. Role in Cancer

Loss of functional pRb protein causes dysregulation of the cell cycle, which contributes to cellular proliferation, a critical biochemical event in the multi-step process of carcinogenesis. *RB/p105* inactivation occurs because of chromosomal deletions, point mutations, frameshifts, and promoter hypermethylation (79, 80). These alterations result in either loss of pRb

expression or, more commonly, expression of a truncated form of pRb caused by premature termination of protein synthesis (69). The truncated form of pRb cannot bind E2F, allowing uncontrolled E2F-mediated transcription of target genes. Another mechanism of pRb inactivation is found in cells infected with HPV (human papilloma virus), a virus causally involved in cervical cancer. HPV-infected cells express the viral coded protein E7, which binds directly to pRb and prevents E2F binding, thus resulting in uncontrolled E2F-mediated transcription of target genes (81-83).

The loss of functional pRb in retinal cells (retinoblasts) results in retinoblastoma cancer (69, 84). There are two main forms of the retinoblastoma cancer (84-86). One form is familial retinoblastoma (hereditary), in which one *RB/p105* allele is inactivated due to a germline mutation, and therefore this inactivation occurs in all the cells of the body. The second allele is inactivated by a somatic mutation occurring during embryonal development or shortly after birth. Familial retinoblastoma is characterized by tumors forming in both eyes (bilateral), with tumor onset occurring in the first eye at approximately ten months of age and in the second eye four years later. In addition, familial retinoblastoma is associated with an increased predisposition to secondary malignancies, particularly sarcomas, later in life (87, 88). The increased chance of secondary malignancies is likely associated with the demonstration that *RB/p105* is mutated in several other cancers (70). Although the loss of function of *RB/p105* in retinal cells is sufficient for retinoblastoma cancer, in other tissues additional genetic

alterations are necessary for tumor formation (89). The second form of retinoblastoma is sporadic (non-hereditary). In these cases, retinoblastoma occurs because of somatic mutations to both alleles of the *RB/p105* gene. Sporadic retinoblastoma is normally characterized by tumors forming in only one eye (unilateral), with a later onset than the familial form of about eighteen months, and a lower incidence of secondary malignancies.

c. Cancer Therapeutic Approaches

With a relatively early diagnosis of retinoblastoma, current chemotherapies, using cyclophosphamide, vincristine, or doxorubicin in conjunction with radiation therapy, are very successful (90). In addition, experimental gene therapy strategies are being developed using retrovirus-mediated delivery of wild-type *RB/p105* (91, 92). Other potential experimental approaches include the manipulation of E2F phosphorylation status with Cdk inhibitors (93, 94). Many of these strategies are being tested in conjunction with chemotherapy.

2. The *p53* Tumor Suppressor Gene

p53 is the most commonly mutated gene in the carcinogenesis process. It is inactivated in over 50% of all human tumors (67, 68). The loss of *p53* function is found in almost all cancer types (67, 68). Thus, *p53* is a potential important target for therapeutic approaches. The *p53* gene product, p53, was first

described in 1979 as a protein that bound to the T-antigen in SV40 transformed cells (95, 96). *p53* was later designated to be a TSG that functions to regulate the cell cycle by activating the expression of genes involved in G_1 checkpoint control upon DNA damage (97). Because of its critical function in response to DNA damage and cell cycle control, *p53* has been called the "guardian of the genome" (98).

a. Function

p53 is a transcription factor that acts as a cell cycle checkpoint guardian, controlling growth arrest, apoptosis, and senescence (99, 100). Under normal conditions, there are low levels of transcriptionally-active p53 present in the cell (100). These low levels are the result of the relatively short half-life of p53 (twenty to thirty minutes) (101). In the event of DNA damage, there is a rapid elevation of transcriptionally active p53 by stabilization of the protein (101, 102). Stabilization of p53 occurs by direct phosphorylation and acetylation modifications, which result in the formation of transcriptionally-active p53 tetramers (101). Some of the proteins involved in p53 activation have been identified. The protein kinase ATM recognizes DNA damage, such as double strand breaks (11, 12). ATM can phosphorylate p53 directly, or it can phosphorylate and activate the protein Chk2, which in turn can activate p53 (103, 104). ATM and Chk2 phosphorylate p53 at a position essential for MDM2 binding, thereby preventing MDM2 binding to p53 (103, 105). MDM2-bound p53

is a target for degradation pathways (discussed below); therefore, prevention of MDM2 binding to p53 stabilizes p53 and increases its half life (105). Another mechanism of p53 activation involves $p14^{ARF}$ (78, 106). Expression of $p14^{ARF}$ can occur by E2F-, Myc-, or Ras-mediated transcription. $p14^{ARF}$ can bind to MDM2, sequestering it from p53, thereby preventing MDM2-mediated inactivation of p53.

Following activation, p53 can act via several mechanisms to halt the cell cycle (100, 107). The most common mechanism is that the transcriptionally-active p53 tetramer binds to specific DNA elements in promoter regions of target genes to initiate transcription (107, 108). One such gene that is activated by p53 is *p21* (109). The *p21* protein product, p21, can bind to cyclin/Cdk complexes, which prevents these complexes from phosphorylating pRb. As noted above, if phosphorylation of pRb does not take place, the transcription factor E2F is not released and cells arrest in G_1, just prior to S-phase. p21 also binds to, and thus inactivates, PCNA (proliferating cell nuclear antigen), which is required for DNA replication (110). Another gene that is activated by p53 is *GADD45* (growth arrest DNA damage), which, like p21, binds to PCNA to inhibit DNA replication (111). By blocking the cell cycle and inhibiting DNA replication, p53 provides time for DNA repair. If the damage cannot be repaired, p53 induces the apoptotic pathway via activation of Bax and Fas/Apo-1 expression (101). Bax is an apoptosis-inducing member of the Bcl-2 protein family and Fas/Apo-1 is a direct activator of the caspase pathway (101, 112). Thus, p53 activation results

in the transcriptional induction of genes to either halt the cell cycle and/or induce apoptosis.

The inactivation of p53 occurs by ubiquitin-mediated proteolysis (102). Proteolysis is initiated by the enzyme MDM2 which attaches the small peptide ubiquitin to p53. Ubiquitination targets a protein for proteolysis, resulting in its degradation (113). As noted above, MDM2-mediated inactivation of p53 is prevented by phosphorylation events by ATM and Chk2 (103, 105). Interestingly, p53 is a transcription factor for MDM2 (114). Thus, the regulation of p53 includes a negative feedback loop in which increased levels of p53 stimulate transcription of MDM2, in turn initiating p53 degradation.

b. Role in Cancer

Many cancer cells exhibit a loss of cell cycle checkpoint regulation (115, 116). Dysregulation of the cell cycle (and loss of induction of apoptosis) can be caused by the loss of functional p53 protein. The most common mechanism for loss of p53 protein function is point mutations in the *p53* gene (117-119). These genetic mutations can result in p53 protein with mutations in the DNA binding domain or the tetramerization domain of the protein. Mutations to the DNA binding domain results in the inability of p53 to bind to specific DNA elements in the promoter region of target genes. Mutations to the tetramerization domain prevents formation of transcriptionally-active p53 tetramers. Similar to pRb inactivation, p53 inactivation can also occur through HPV infection. HPV-infected

cells express the viral encoded protein E6 that binds to and inactivates p53 (the E7 viral protein inactivates pRb) (99, 117). Additional mechanisms of inactivation of p53 are indirect, such as multiplication of the *MDM2* gene or inactivation of the $p14^{ARF}$ gene, resulting in a decrease in the half-life of p53 (99, 117). All of the above mechanisms of p53 inactivation might result in loss of cell cycle checkpoint control, resulting in insufficient time necessary for DNA repair, thereby increasing the occurrence of mutations that can arise from damaged DNA. In addition, loss of induction of apoptosis due to p53 inactivation might result in accumulation of cells with DNA damage and/or mutations.

Germline mutations to a single allele of the *p53* gene are associated with the rare syndrome, Li-Fraumeni (L-F) (117, 120). L-F is an autosomal-dominant disorder that features the onset of tumors under the age of forty-five. Individuals affected by L-F are predisposed to several types of cancer, including sarcomas, carcinomas of the breast, brain, and adrenal gland, and acute leukemia (121). Most *p53* mutations occurring in L-F are missense germline mutations in the DNA binding domain of the gene (117).

c. Cancer Therapeutic Approaches

The cytotoxic effect of many radiation and chemotherapeutic strategies on cancer cells depends on activation of the apoptosis pathway (106, 122). Cells in which the *p53* gene is inactivated are often drug resistant because the p53-induced apoptosis pathway cannot be activated (123, 124). Several therapeutic

approaches have been developed to increase the half-life of or reactivate p53. These include the introduction of functional *p53* by gene therapy, the use of monoclonal antibodies to prevent MDM2 binding and subsequent degradation of p53, and the use of pharmacological agents that directly bind to mutated p53, causing structural changes in the p53 protein that allow it to form transcriptionally-active tetramers (125). Another therapeutic approach is the use of bioengineered adenoviruses such as ONYX-015 (126-129). ONYX-015 is a mutated variant of the human group C adenovirus that specifically replicates in tumor cells with mutated p53, resulting in cell lysis. ONYX-015 is currently in the initial phases of human trials.

B. Oncogenes

Oncogenes are mutant forms of normal cellular genes (proto-oncogenes) that are expressed in tumor cells and are responsible for some of the transformed characteristics of these cells. When proto-oncogene expression or its' gene product is altered, such a gene can be become an oncogene. The conversion of a proto-oncogene to an oncogene can occur by point mutations, gene amplification, or chromosomal rearrangements (1, 130). Any of these mechanisms might result in an increase in protein expression or expression of a functionally-active protein product. As oncogenes are dominant-acting, only a single allele of a proto-oncogene needs to be altered to render it oncogenic. Oncogenes code for growth factors, receptor tyrosine kinases, non-receptor

tyrosine kinases, GTP-binding proteins, cytoplasmic serine/threonine kinases, and transcription factors (84). Oncogenes were first discovered in viruses (131-134). There are now over one hundred known human oncogenes, many of which are expressed in specific cancer types (1). Two "classical" oncogenes, *RAS* and *MYC*, are discussed in detail below.

1. The *RAS* Family of Oncogenes

The *RAS* gene family consists of three members, *H-RAS*, *K-RAS*, and *N-RAS* (135-137). They are members of the Ras superfamily of small GTPases, which are involved in signal transduction pathways that regulate cell growth and differentiation (138). *H-RAS* was the first human oncogene that was cloned and characterized (139-142), and was demonstrated to be homologous to the *RAS* gene of the Harvey murine sarcoma (*v-H-RAS*) (142). Oncogenic forms of *RAS* are implicated in approximately 30% of all human cancers (143).

a. Function

The *RAS* gene product (Ras) is a cell membrane-bound guanosine triphosphate (GTP)/guanosine diphosphate (GDP)-binding protein that, upon activation, transduces signals from the cell membrane via several effector pathways to the nucleus resulting in cellular proliferation, differentiation and apoptosis (135). Ras is expressed in the cytoplasm where it undergoes post-translational modifications that are required for Ras localization to the cell

membrane and activation (138). These modifications include prenylation, proteolysis, methylation, and palmitoylation (135).

The first post-translational modification that occurs to Ras is prenylation (135, 137). Prenylation involves the covalent addition of either a 15-carbon farnesyl or 20-carbon geranylgeranyl group to a conserved carboxy-terminal cysteine residue of Ras (135). This addition occurs by farnesyl protein transferase or gerenylgerenyl pyrophosphate and is important in targeting Ras to the cell membrane (144) and Ras interactions with other proteins (145). After prenylation, proteolytic cleavage occurs to specific amino acids in the carboxy-terminal by zinc metalloproteinases and the carboxyl group of the prenylated cysteine residue is then methylated (137, 146). The final post-transcriptional modification is the addition of lipid groups by palmitoylation to cysteines near the prenylated carboxy-terminus (147). Palmitoylation occurs by a palmitoyltransferase and causes an increased affinity of Ras to the cell membrane due to an increase in the electrostatic interactions with negatively charged groups on the inner cell membrane surface (148). Although the prenylation and proteolysis steps are irreversible, the methylation and palmitoylation steps are reversible and hypothesized to play a regulatory role in Ras activation (149).

The membrane-bound Ras is normally in its GDP-bound, inactive form, due to rapid hydrolysis of GTP to GDP by intrinsic Ras GTPase ability and GTPase-activating proteins (GAPs) (138, 150, 151). Ras is activated by

extracellular signals as a result of growth factors binding to their associated receptors. Examples of activating growth factors include EGF (epidermal growth factor), TGF-α (transforming growth factor-α), and PDGF (platelet-derived growth factor) (135). The binding of these growth factors to their receptor causes receptor dimerization and activation of an intrinsic tyrosine kinase. The tyrosine kinase activity results in autophosphorylation of specific tyrosine residues located on the intracellular region of the receptor. The phosphorylated tyrosine residues then interact with SH2 (Src homology 2) domains of the adapter protein Grb2 (135). Grb2 also contains two SH3 (Src homology 3) domains that, in turn, interact with proline-rich regions of the cytosolic protein, SOS (son of sevenless) (135). The interaction between Grb2 and SOS allows for the recruitment of SOS to the cell membrane, in near proximity to Ras (138). SOS is a guanine nucleotide exchange factor that binds to Ras causing a conformational change in the Ras protein, resulting in GDP dissociation and binding of GTP (152, 153). Activated GTP-bound Ras can then activate several distinct effector pathways that are involved in transmitting the membrane signal to the cell nucleus. These pathways include the serine-threonine kinase Raf-1 and PI3-kinase pathways. The best-characterized pathway is the Raf-1-MEK-ERK pathway (154). Activated Ras recruits the serine-threonine kinase Raf-1 (mitogen activated protein kinase kinase kinase; MAPKKK) to the membrane through an interaction with a Ras-binding domain located in the N-terminal of Raf-1. The binding of Raf-1 to Ras causes a structural change in the Raf-1 protein allowing it to be

phophorylated by various kinases, resulting in Raf-1 activation. Activated Raf-1 then phosphorylates and activates MEKK (MAP/ERK kinase kinase), which subsequently phosphorylates and activates ERK (extracellular signal regulated kinase; MAP/ERK kinase; MEK). ERK then translocates to the nucleus where it can phosphorylate and activate several growth-related transcription factors, such as c-Myc, c-Fos, c-Jun, and ELK-1 (155).

Activated Ras can also bind to and activate the catalytic domain of PI3-kinase (156, 157). PI3-kinase activation leads to an increase in the concentration of 3'-phosphorylated inositol lipids. These lipids can then activate the GTP/GDP proteins Rac and Rho (135, 158, 159). Rac and Rho are regulators of actin cytoskeletal reorganization and are important in the cell transformation process (138). Although both proteins play an important role in cell morphology, cell motility, cell adhesion, and cytokinesis, each one regulates a different aspect of these processes. Rac is involved in regulating ruffling and lamellipodia formation, while Rho is involved in regulating stress fiber formation and focal adhesions (138). PI3-kinase can also activate the serine-threonine kinase Akt (157), which is involved in suppression of the apoptotic pathway by inhibiting the expression of pro-apoptotic proteins Bad and Caspase 9 (160).

Although Ras plays a major role in cellular proliferation and differentiation, there is evidence that Ras also causes antiproliferative effects and induction of apoptosis. Overexpression of oncogenic Ras can induce p21 (161), p53, and p16 expression (162) resulting in G_1 arrest. Overexpression of oncogenic Ras

has also been shown to induce cellular apoptosis, mediated through the activation of the pro-apoptotic protein, caspase 8 (163). Thus, Ras is a multi-functional protein that is involved in activation of cellular signaling cascades, which induce proteins that regulate cell proliferation, differentiation, and apoptosis.

b. Role in Cancer

The *RAS* gene is mutated in 30% of all cancers, resulting in a protein product that has the inability to hydrolyze GTP (143). Thus, the GTPase activity of Ras is abolished, resulting in constitutive expression of activated Ras and downstream effector pathways, which results in continuous transcription of genes involved in cellular growth, differentiation, and transformation. Mutations in *RAS* genes that cause the expression of activated protein are single point mutations and have been localized to amino acids 12, 13, 59, or 61 (143). Generally, only one of the three *RAS* genes is involved in a specific type of cancer. Oncogenic *K-RAS* is the most occurring *RAS* member in cancer, while *H-RAS* is the least occurring (135).

Early studies involving *H-RAS* demonstrate that the oncogenic form directly transforms various rodent and human cell strains in culture (139, 164-166). Although it was originally reported that the oncogenic form could malignantly transform finite lifespan rodent fibroblasts in culture (164), recent studies have demonstrated that additional genetic alterations (which involve the

inactivation of the p53 and pRb pathways, or activation of other oncogenes) are required for the *H-RAS* oncogene to malignantly transform both diploid rodent and human cells in culture (38, 167-171).

c. Cancer Therapeutic Approaches

As *RAS* is the second most commonly mutated gene in human cancer (*p53* is the first), the development of Ras inhibitors as potential anticancer agents has attracted considerable attention. Three main approaches have been employed to prevent the actions of oncogenic *RAS* and Ras protein. These include inhibition of *RAS* expression (by antisense oligonucleotides); inhibition of Ras post-translational modifications by farnesyl transferase, geranylgeranyl transferase and prenylated protein methyltransferase; and inhibition of Ras downstream effectors by Raf-1 and ERK kinase inhibitors (135-137). Several of these approaches are in various clinical trial phases, while many others are in pre-clinical trials.

2. The *MYC* Family of Oncogenes

The *MYC* gene family consists of three members, *c-MYC*, *N-MYC*, and *L-MYC* (172, 173). *c-MYC* was the first *MYC* gene discovered (174). It was discovered by its homology with the oncogene *v-MYC*, which is carried by the avian myelocytomatosis virus strain MC29. *MYC* overexpression has been found in many cancers (175), including breast and colon cancers (176, 177).

a. Function

The *MYC* gene product is a transcription factor involved in promoting cell proliferation, growth, and differentiation (178). The Myc protein is synthesized in the cytoplasm and translocated to the cell nucleus; it is continuously expressed in cycling cells. It has a basic helix-loop-helix-zipper (bHLHZ) domain, a characteristic of many transcription factors involved in DNA binding and protein-protein interactions (179, 180). Although the Myc protein can bind to DNA directly, it has very weak transcriptional activity and heterodimerizes with the protein Max (which also has a bHLHZ domain) to form a transcriptionally-active protein complex (178). The Myc-Max heterodimer binds to specific DNA promoter elements to initiate transcription of many genes involved in cell growth control (175). Max, like Myc, has little transcriptional activity on its own. In addition, Max also heterodimerizes with Mad, a protein that antagonizes the functions of Myc (178). Not only does Mad sequester Max away from Myc, but the Mad-Max heterodimer also competes for binding with the Myc-Max heterodimer resulting in suppression of Myc transcriptional activity. *MYC* expression is induced by growth factors, cytokines, and mitogens. Many of these activate the Ras-Raf-1-MEK-ERK signaling cascade to induce expression of transcription factors such as c-Fos, c-Jun, and NF-κB, all of which have been shown to regulate *MYC* expression and transcription (181-183).

b. Role in Cancer

Myc overexpression plays a causal role in many aspects of the carcinogenesis process, such as cell proliferation, immortalization, and angiogenesis. Overexpression of Myc occurs mostly by genetic rearrangements, such as *MYC* gene amplification and chromosomal translocations (173). For example, in Burkitt's lymphoma, a characteristic chromosomal translocation occurs, which fuses the *c-MYC* gene with various immunoglobulin genes located on other chromosomes (184). This translocation causes the *c-MYC* gene to be under control of a continuous, transcriptionally-active immunoglobulin gene.

Myc promotes cell proliferation by inducing expression of cyclin D1 and D2 (185, 186), and Cdk4 (187), thereby shortening G_1 and promoting entry into S-phase. Myc-induced expression of cyclin D2 sequesters the $p27^{KIP1}$ Cdk inhibitor and p21, further prolonging the activation of Cdks (185-188). As described above, the activation of cyclin/Cdk complexes are involved in phosphorylating pRb, thus activating E2F-mediated transcription, which further promotes G_1 to S progression (76).

Myc overexpression also plays a role in a cancer cell's acquisition of infinite proliferative capacity, termed cellular immortalization (a key process in carcinogenesis; discussed in greater detail in Section III). *MYC* was thought to be an "immortalizing gene" since transfection and overexpression of the gene in rat embryo fibroblasts in culture was reported to result in immortalization (170, 171, 189). Recent studies showed, however, that Myc caused cellular

immortalization indirectly, through activation of the *hTERT* gene (human telomerase reverse transcriptase), the catalytic subunit of the telomerase enzyme (190-193). Telomerase is involved in the maintenance and regulation of telomere length and has been shown to be important in cellular immortalization (194).

In addition to proliferation and cellular immortalization, Myc overexpression has also been shown to be involved in cellular transformation and tumor angiogenesis. Although the mechanisms are not understood, there is a strong correlation between increased levels of Myc expression and decreased expression of proteins involved in cell adhesion (ex. collagen), cytoskeletal structure (ex. fibronectin), and extracellular matrix (ex. tropomysin) (175, 195). Increases in Myc expression have also been correlated with decreases in THSB1 (an inhibitor of angiogenesis) expression and increases in vascularization (angiogenesis) (196-198).

c. Cancer Therapeutic Approaches

Several *MYC* therapeutic approaches are currently being investigated. One possible approach is the use of *MYC* antisense oligonucleotides. Experimental studies have shown that the use of *MYC* antisense oligonucleotides inhibits *MYC* mRNA translation and prevents cells from entering S-phase (199, 200). Another variation of antisense therapy is the use of triple helix-forming oligonucleotides (TFO) targeted to regions of the *MYC* gene

promoter (201, 202). TFOs act directly on the gene by binding to double-stranded DNA in a stable, sequence-specific manner, preventing transcription factor binding and thus, transcription of *MYC*.

III. Acquired Characteristics of Cancer Cells

The outcome of the genetic alterations involved in the multi-step process of carcinogenesis is that cells acquire the functions necessary for tumor formation. These functions include growth stimulatory signal autonomy, insensitivity to growth inhibitory signals, infinite proliferative capacity (cellular immortalization), angiogenic potential (the ability to induce new blood vessels and capillaries), and the ability to invade normal tissue and metastasize (85, 203).

A. Growth Stimulatory Signal Autonomy

Normal cell proliferation occurs in response to mitogenic growth factors. These growth factors bind to receptors and activate a variety of intracellular signaling molecules involved in cell growth and proliferation. Examples of growth factors include EGF and PDGF. There are several methods by which cancer cells can acquire growth stimulatory signal autonomy. One such mechanism is the cancer cell's acquisition of the ability to produce growth factors to which it is responsive (autocrine stimulation). For example, PDGF stimulates cell growth, proliferation, and motility through binding to and activating the PDGF-β tyrosine kinase receptor (204). In cancers such as gliomas and sarcomas, PDGF expression has been shown to be up-regulated (205). A second mechanism by which cancer cells acquire growth stimulatory signal autonomy is overexpression of growth factor receptors. This leads to an amplification of the signal

transduction pathways involved in cell growth and proliferation. It has been shown that the EGF receptor (EGFR) is overexpressed in a variety of cancers (205, 206), and that this overexpression is caused by EGFR gene amplification (206-208). EGFR is a tyrosine kinase receptor that is activated by several growth factors. Overexpression of EGFR is associated with increased growth rate, angiogenesis and metastasis, as well as inhibition of apoptosis (209). A third and most common mechanism in acquisition of growth stimulatory autonomy is alteration of the signal transduction pathways leading to cell growth and proliferation. A classical example is the oncogenic *RAS* gene (discussed previously in Section II), which produces a protein that is structurally altered, causing continuous activation of downstream mitogenic signal transduction pathways without the binding of a growth factor to a receptor (135). Besides alterations in the initiation of signal transduction pathways, there can also be alterations in effector genes within these pathways. For instance, the *MYC* gene is translocated in several types of cancer, resulting in overexpression and continuous progression through the cell cycle (discussed previously in Section II) (173, 178).

B. Insensitivity to Growth Inhibitory Signals

Normal cellular homeostasis requires a balance between proliferative signals and growth inhibitory signals. Therefore, the mechanisms involved in acquired growth stimulatory signal autonomy often contribute to an insensitivity to

antigrowth signals. Inhibitory signals block cell proliferation via receptors that couple to intracellular signaling molecules, similar to the mechanism for growth stimulation. However, the intracellular signaling molecules for growth inhibition possess opposite functions than the molecules for growth stimulation (e.g., a phosphatase versus a kinase).

One mechanism of acquired insensitivity to growth inhibitory signals involves TGF-β (transforming growth factor-β). TGF-β is a growth factor that has both stimulatory and inhibitory effects on cell cycle progression, which are cell type specific. For example, it has been shown that TGF-β acts as a growth factor in fibroblasts and a growth inhibitor in epithelial and endothelial cells (210). Its' inhibitory effects are mediated through several signal transduction pathways (211, 212). One such pathway involves pRb (211, 213). In normal epithelial cells, TGF-β-responsive effectors inhibit the phosphorylation of pRb (and therefore the release of E2F) by inhibiting the Cdks involved in this process, thus blocking cell cycle progression. In some cancer types, such as retinoblastoma, direct mutations to *RB/p105* result in the inability of pRb to respond to growth inhibitory signals such as TGF-β, which causes continuous cell proliferation mediated through E2F (69). In many other cancer types, such as lung, breast, colon, and skin cancer, down-regulation of TGF-β receptors or mutations in TGF-β-downstream effectors occurs (214).

Another important gene that contributes to an insensitivity to antigrowth signals is the *p53* gene (discussed previously in Section II). p53 responds to

growth inhibitory signals to activate various pathways, which can result in either cell cycle arrest or apoptosis (99, 100). In several cancers, the *p53* gene is often mutated, resulting in a lack of responsiveness to these signals.

C. Infinite Proliferative Capacity (Cellular Immortalization)

Although increased responsiveness to growth signals and/or decreased responsiveness to inhibitory signals contribute to the malignancy of cancer cells, most cancer cells must acquire an infinite proliferative capacity (cellular immortalization) to allow for expansive tumor growth. Based on *in vitro* studies, normal human cells have a finite lifespan. Several normal human cell types, when grown in culture, have the capacity to double between 40-70 times before they enter a terminal stage termed senescence (215, 216). Senescent cells are viable and metabolically active, but they can no longer divide. In contrast, many human tumor-derived cell strains do not senesce; they can proliferate indefinitely.

In normal human cells, the cellular aging mechanism is correlated with the loss of telomeric ends (217). Telomeres are nucleoproteins at the ends of chromosomes that function to maintain chromosome stability by protecting against degradation, rearrangement, and fusion with other chromosome ends (218). Human telomeres are made up of repetitive DNA (a hexanucleotide, TTAGGG), and shorten by 50-200 nucleotides at each cell division. This shortening is caused by the "end-replication problem," which occurs because the lagging strand of DNA synthesis cannot replicate the extreme 3' end of the

chromosome (219). When the telomeres reach a critical short length, cellular senescence results (220, 221). Telomere shortening has been shown to be associated with genomic instability (194, 222). Senescence induced by telomere shortening is now assumed to be a tumor suppression mechanism since it blocks the further replication of cells that may have lost critical genes, as well as the accumulation of mutations associated with genomic instability (217).

Telomere length is maintained by telomerase, an enzyme normally expressed in stem cells and reproductive cells (218, 223). Approximately 85% of all tumor types have telomerase expression; whereas most normal tissues lack telomerase activity (224, 225). Telomerase expression is necessary for most cell types *in vitro* to overcome senescence and become immortal. The introduction of the gene for the catalytic subunit of telomerase, *hTERT*, into normal human fibroblast cells results in telomere lengthening and cellular immortalization (226, 227). Although hTERT expression alone was sufficient to immortalize normal human fibroblast and retinal pigment epithelial cells (226-228), one study using normal human keratinocytes and retinal pigment epithelial cells showed that, besides hTERT expression, other genetic alterations (including inactivation of *p53* and *RB/p105*) were required for cellular immortalization (229). The 15% of tumor types that do not possess telomerase activity maintain telomere stability by a telomerase-independent mechanism called alternative lengthening of telomeres (ALT) (230). Recent evidence suggests that the mechanism involved in the ALT pathway involves telomere recombinational events (231).

D. Angiogenic Potential

To function properly, normal cells need sufficient oxygen and nutrients, which are supplied by the vasculature *in vivo*. A normal cell must be located within 100-200 μm of a capillary to survive *in vivo* (232). Tumors cannot grow beyond a critical size or metastasize without a blood supply. Cancer cells have overcome this problem by acquiring the ability to activate angiogenesis, the process by which new blood vessels are formed via the invasion of endothelial cells from existing blood vessels in response to extracellular signals (233). One of the main angiogenic regulators is VEGF (vascular endothelial growth factor), which is a specific mitogen for vascular endothelial cells (234, 235). VEGF binds to transmembrane tyrosine kinase receptors resulting in activation of signal transduction pathways involved in endothelial cell migration and proliferation. In normal cells, there is an equilibrium between positive angiogenic regulators, such as VEGF, and negative angiogenic regulators, such as THBS1 (thrombospondin-1) (62, 236). THBS1 associates with the extracellular matrix and possesses a variety of anti-angiogenic functions, including inhibition of endothelial cell proliferation, migration, and morphogenesis. In cancer cells, the equilibrium is shifted by an increase of the expression of positive angiogenic regulators and a decrease in the expression of negative angiogenic regulators (237). The result of this imbalance is increased proliferation of nearby endothelial cells, which migrate into the growing tumor. It is has been shown that the ability to induce

angiogenesis occurs as a mid-stage event in tumor development, prior to tissue invasion (233).

E. Tissue Invasion and Tumor Metastasis

Fully malignant cells, besides having acquired all the properties detailed above, are invasive and can be metastatic. Malignant cell invasion is the active process by which such cells translocate into and through extracellular matrix barriers (238). Metastasis is the process by which malignant cells depart from the primary tumor and travel to distant sites within the body to form secondary tumors (239). Although the genetic alterations involved in tumor initiation and formation vary between different cancer types, the alterations required for tumor invasion and metastasis are similar. There are several steps involved in tumor invasion and metastasis, each requiring the expression of different proteins involved in cell to cell adhesion, cell-matrix interaction, proteolysis, and motility (239). In normal cells, there is a balance between the expression of these proteins. In cancer, this balance is shifted; pro-invasive and pro-metastatic genes are up-regulated and anti-invasive and anti-metastatic genes are down-regulated.

1. Tissue Invasion

The first steps involved in malignant cell tissue invasion are the acquired abilities to attach to the extracellular matrix components and disrupt the

basement membrane (84). Both involve changes in the expression of integrins and CAMs (cellular adhesion molecules). CAMs are involved in cell to cell adhesion interactions and produce anti-metastatic signals through these interactions (E-cadherin is one such example of a CAM) (240). In metastasis, the loss of CAM functions causes a decrease in cellular adhesiveness. Integrins are transmembrane receptors that bind to extracellular matrix components such as laminin, fibronectin, and collagens (241). The binding of integrins to these matrix components activates signal transduction pathways, which activate genes involved in cell cytoskeletal organization and support. Integrins consist of various α and β subunits, and different combinations of these subunits result in different integrin subtypes. Each integrin subtype has a distinct substrate preference and thus, different integrin combinations are expressed in normal cells, while other combinations are expressed only in malignant cells (242, 243). The combination of loss of CAM function and changes in integrin subunit expression are the initial steps in malignant cell invasion.

Upon disruption the basement membrane, malignant cells translocate across the extracellular matrix barriers by proteolysis and migration (84). This translocation involves the expression of MMPs (matrix metalloproteinases), such as collagenases, and gelatinases (244-246). These proteases are up-regulated in invasive tumors and allow the tumor cells to cleave and degrade the extracellular matrix components. In normal cells, the expression of TIMPs (tissue

inhibitors of metalloproteinases), inhibit MMPs by direct binding resulting in inactivation (245).

In addition to an ability to degrade the extracellular matrix, malignant cells must have the ability to migrate through the matrix. There are several factors that contribute to this migration by stimulation of cell motility. These factors, such as HGF/SF (hepatocyte growth factor/scatter factor) (247) and IGF-1 (insulin-like growth factor type 1) (248), can be secreted by the tumor cells and function in an autocrine manner. In conjunction with cell secreted factors, the components of the extracellular matrix (laminin, fibronectin, and collagen) also act as motility factors (238, 249). As the components of the extracellular matrix are cleaved by MMPs, the resultant matrix protein fragments act as chemotaxis signals, which can further stimulate tumor cell migration.

2. Metastasis

Metastasis is the process by which malignant cells depart from the primary tumor and travel to distant sites within the body to form secondary tumors (239). Tumor metastasis is the leading cause of death in cancer patients. Normally, metastasis occurs via the blood circulation, but it can also occur through the lymphatic system (84). Interestingly, not all the cells within a primary tumor can metastasize (250). This is consistent with the multi-step process of carcinogenesis, which suggests that additional genetic alterations are required for tumor metastasis.

Malignant cells that have detached from the primary tumor and penetrated and migrated through the extracellular matrix and basement membrane (as detailed above) can enter blood capillaries or lymphatic vessels. Invasion into the transport systems occurs by similar mechanisms as noted above for malignant cell migration through the extracellular matrix barriers (239, 245, 246). The expression of MMPs allows malignant cells to gain access to small vessels by proteolytic degradation, which produces an opening in the outside wall of the vessel through which tumor cells can migrate into the lumen of the vessel (84). These cells can then be transported to other sites of the body. Within the circulation, the cells adhere to one another, to lymphocytes, and to platelets (84), forming emboli that can adhere to the inner surface of capillaries. Upon attachment to the inner surface of the capillary, MMPs break down the vessel wall, and the malignant cells can actively migrate into the intercellular matrix of the surrounding tissues and resume tumor growth, resulting in secondary tumors.

IV. Models of the Multi-step Process of Carcinogenesis

A. Colorectal Cancer: An *In Vivo* Model for Carcinogenesis

Colorectal cancer is the second leading cause of cancer death (lung cancer is the first) in the United States. It is estimated that one out of seventeen people will develop this form of cancer in their lifetime and 139,000 people will be diagnosed with colorectal cancer in the United States this year (251). Ninety-five percent of all colorectal cancers occur sporadically, while the remaining five percent result from the hereditary predispositions; Familial Adenomatous Polyposis (FAP; one percent) and Hereditary Nonpolyposis Colorectal Cancer (HNPCC; four percent) (252). It is estimated that one out of every 10,000 people have a hereditary predisposition for FAP or HNPCC, and over eighty percent of these individuals will eventually develop colorectal cancer (253). Risk factors for colon cancer include age, weight, and diet, but people with inherited genetic alterations are much more susceptible.

The genetic alterations involved in FAP have been well characterized, making this form of colorectal cancer the best understood *in vivo* example of the multi-step process of carcinogenesis (27, 30-33). Because FAP develops in distinct stages from pre-tumorigenic lesions, to benign tumors, to malignant tumors, tissue can be obtained at the various stages for study (31). The onset of FAP is characterized by the presence of microscopic epithelial lesions in the colon called dysplastic aberrant crypt foci (ACF). ACF can result in the formation of thousands of colonic adenomas (benign tumors). The higher the number of

adenomas, the greater the probability that one of these adenomas will result in a malignant tumor. Carcinogenesis of colorectal cancer involves several genetic alterations that result in proto-oncogene activation and TSG inactivation. Based on observations in individuals with a high risk for colon cancer (FAP or HNPCC), it is hypothesized that there are a minimum of seven independent genetic alterations that are required for a single cell to acquire the ability for colorectal carcinoma formation (27). These specific genetic alterations are generally associated with different stages of colorectal tumor formation and progression (27). The accumulation of these specific alterations in a single cell results in tumor formation and progression (27).

In all cases of FAP and 80% of sporadic colorectal cancer, the initial genetic alteration is a mutation of the adenomatous polyposis coli (*APC*) gene that results in inactivation (254). Individuals that have a germline mutation to the *APC* gene are predisposed to FAP. The *APC* gene is located on chromosome 5q21 (255) and encodes for a protein that is involved in signal transduction, cellular adhesion, stabilization of the cytoskeleton, and possibly in the regulation of the cell cycle and apoptosis (254). Inactivation of this gene is an initiating factor in adenoma formation. *APC* has been defined as a TSG, since both alleles of this gene must be inactivated for loss of function to occur (256). Loss of functional *APC* often occurs by frameshift or nonsense mutations, resulting in the expression of an inactive truncated protein product (253). The inactivation of this gene is sufficient for the occurrence and growth of multiple early colorectal

adenomas found in FAP and most cases of sporadic colorectal cancer (257). Genetic instability is commonly associated with adenoma formation and development (258, 259). Although the exact mechanism for this instability has yet to be discovered, deficiencies in DNA mismatch repair mechanisms have been implicated (27, 260, 261). The genetic alterations that result from this instability can result in cells with a selective growth advantage, promoting the progression to malignancy. Other altered genes commonly found in colorectal cancer include activation of the *K-RAS* proto-oncogene and inactivation of the *p53* and the *DCC* (deleted in colorectal carcinoma) TSGs (27). The oncogenic form of *K-RAS* is found to be present in more than 50% of all colorectal cancers (143). *p53* is located on chromosome 17p, a region lost in 75% of all colorectal cancers (31). The other allele of *p53* is frequently mutated, resulting in complete loss of function of the *p53* gene. The second most common region of chromosome loss occurs at 18q21 where the *DCC* gene is located (31). The *DCC* gene is a candidate TSG that may be involved in regulating apoptosis (262). The *DPC4* (deleted in pancreatic cancer 4, also known as *SMAD4*), a candidate TSG, also located in the 18q21 region, may be involved (263). *DPC4* is a transcription factor involved in the activation of several genes critical in cell cycle control (264). The genetic alterations that are involved in FAP and sporadic colorectal cancer have been hypothesized to follow a multi-step pathway shown schematically in Figure 3 (27, 252, 265).

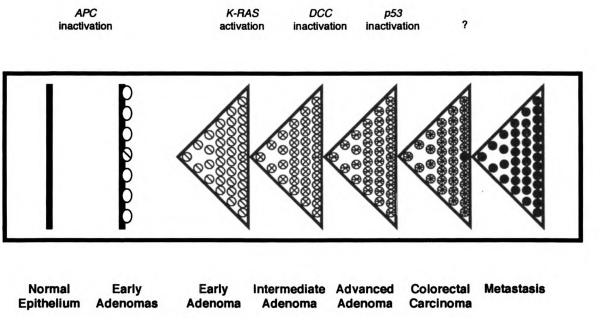

Figure 3. The multi-step process of colorectal cancer. Adapted from (27, 252, 265).

The other heritable form of colorectal cancer is HNPCC. HNPCC and the remaining 20% of sporadic colorectal cancer cases are a result of the "hypermutability pathway" (252). These forms of colorectal cancer result from inherited mutations in mismatch repair genes (266, 267). As noted above in Section I, DNA damage can occur spontaneously because of errors by the polymerase involved in DNA replication (10). The mismatch repair genes are involved in repairing mismatched or unpaired bases in DNA that occur during DNA replication, genetic recombination, or as a result of chemical mutagen exposure. Examples of these genes commonly mutated in HNPCC include *hMLH1* and *hMSH2* (252, 268). Inactivation of these genes results in an increase in mutation frequency and thus, an increase in the probability of a TSG or proto-oncogene will be mutated. Individuals who are heterozygous for any of the mismatch repair genes are predisposed to HNPCC, because a loss of function of the remaining normal allele results in bi-allelic inactivation.

B. The MSU Lineage of Cells: An *In Vitro* Model for Carcinogenesis

To obtain a thorough understanding of the multi-step process of carcinogenesis in human soft tissue sarcomas, experiments were performed in the Carcinogenesis Laboratory at Michigan State University to determine the genes involved in fibrosarcoma formation (malignant fibroblastic tumors) (36, 165, 166, 269). Normal human diploid foreskin derived fibroblasts were used in these experiments for several reasons. First, fibroblasts are found in all organs

of the body, and they can become malignant. Although fibrosarcomas make up only 0.2% of all cancers, soft tissue sarcomas, the larger group to which fibrosarcomas belong, are very similar and make up 1-2% of all cancers (251, 270). Second, at the time of establishing the model, there were several reports on the ability to transform rodent fibroblast (164, 170, 171, 271). Since genes are highly conserved between rodents and humans, this suggested that information on genes and their phenotypic properties in the neoplastic transformation of rodent fibroblasts would be relevant to human fibroblasts. Third, fibroblasts are easy to grow and manipulate *in vitro*. Fibroblasts were the first cell type successfully grown in culture and are the most commonly used cell type utilized in *in vitro* experiments. To validate the use of fibroblasts, studies were carried out to determine appropriate *in vitro* end points to study transformation and a suitable animal system to determine the ability of fibroblasts to form tumors (McCormick *et al.*, unpublished studies) (28). Athymic mice were injected subcutaneously with cells derived from human fibrosarcomas into athymic mice. Sarcomas arose at the site of injection in every case. When normal human fibroblasts were injected in the same manner, no growth of any sort was observed. These findings indicate that if normal human fibroblasts can be transformed to tumorigenic cells *in vitro*, tumor formation in athymic mice is an appropriate experimental endpoint.

1. Generation of the Cell Strain MSU-1.0

Initial studies were performed to transform normal human fibroblasts *in vitro* to malignant cells by transfection with various oncogenes, which included *H-RAS* (166), *N-RAS* (269), and *v-SIS and c-SIS* (165). These studies resulted in cells that did not form tumors when injected into athymic mice. Although the cells had the *in vitro* characteristics of malignantly transformed human fibroblasts, the expression of these properties was somewhat muted. For example, these cells formed colonies in soft agarose, but the colonies were smaller than those formed by malignantly transformed fibroblasts (McCormick *et al.*, unpublished studies). One possible reason for this is that the cells were nearing the end of their replicative life span at the time they were injected into athymic mice and thus did not have the replicative capacity to form tumors. In retrospect, a more plausible reason is that overexpression of an oncogenic *RAS* gene alone is not capable of causing the malignant transformation of human fibroblasts, as had been previously claimed in rodent fibroblasts (164). This explanation is now supported by the findings of Hahn *et al.*, 1999 (168), that oncogenic *H-RAS* cannot directly transform normal human fibroblasts or telomerase positive, infinite lifespan human fibroblasts to malignant cells; additional genetic alterations are necessary. This observation supports the multi-step process of carcinogenesis.

Since human fibroblasts may require an extended lifespan to acquire all the changes needed for tumorigenicity, Morgan *et al.*, 1991 (36), developed an immortal human fibroblast cell strain. This study was based upon previous

findings that normal rat embryo fibroblasts could be immortalized using a plasmid carrying the MC29 *v-MYC* oncogene (170). A recombinant plasmid containing the MC29 *v-MYC* oncogene linked to the geneticin resistance gene was transfected into normal human foreskin-derived diploid fibroblasts, designated LG1 (36). Upon drug selection using the antibiotic geneticin, several clones continued to grow. One clonal population expressing the *v-MYC* oncoprotein was found. After these v-Myc expressing cells were carried in culture for multiple population doublings, the cells senesced, but a small number of cells continued to grow. These cells, designated MSU-1.0, have undergone over 200 population doublings since the time of senescence and are thus considered immortal (36). MSU-1.0 cells are diploid, karyotypically stable (36), and telomerase positive (272).

2. Generation of the Cell Strain MSU-1.1

From MSU-1.0 cells, a spontaneous variant strain with a growth advantage arose in the culture. These cells were isolated and designated MSU-1.1 (36). MSU-1.1 cells are near-diploid, karyotypically stable, and contain 45 chromosomes including two marker chromosomes: a chromosome 1;11 marker and a chromosome 12;15 marker (Figures 4, 5, and 6). These cells contain four genetic alterations as a result of the formation of the two marker chromosomes: loss of DNA fragments on chromosomes 11 (11p15→11pter), 12

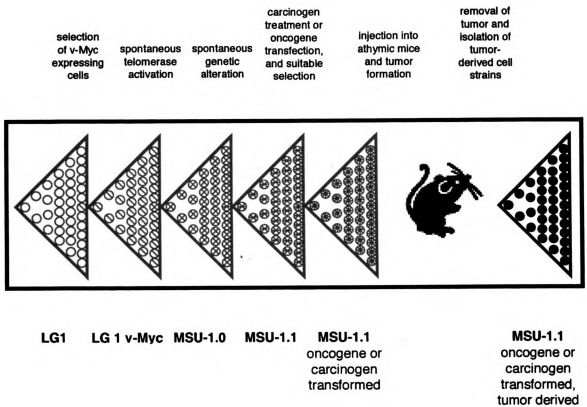

Figure 4. Schematic diagram of the MSU lineage of cells.

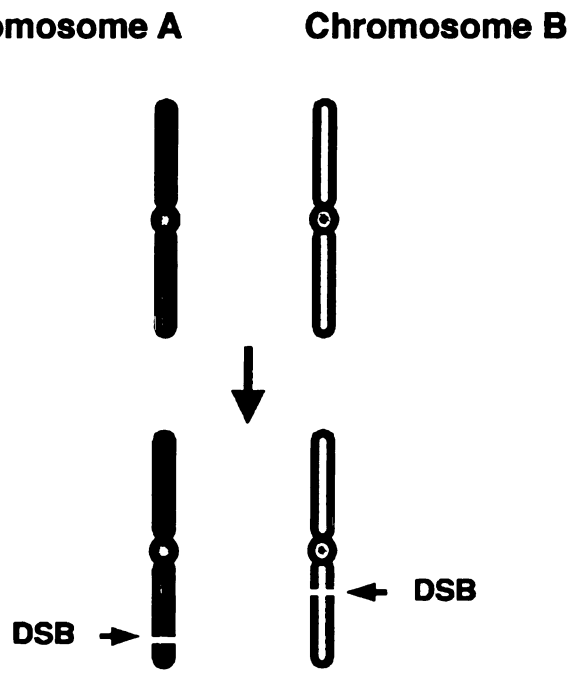

Figure 5. Karyotype analysis of an MSU-1.1 cell showing the two marker chromosomes Marker 1, M1, and Marker 2, M2. (36)

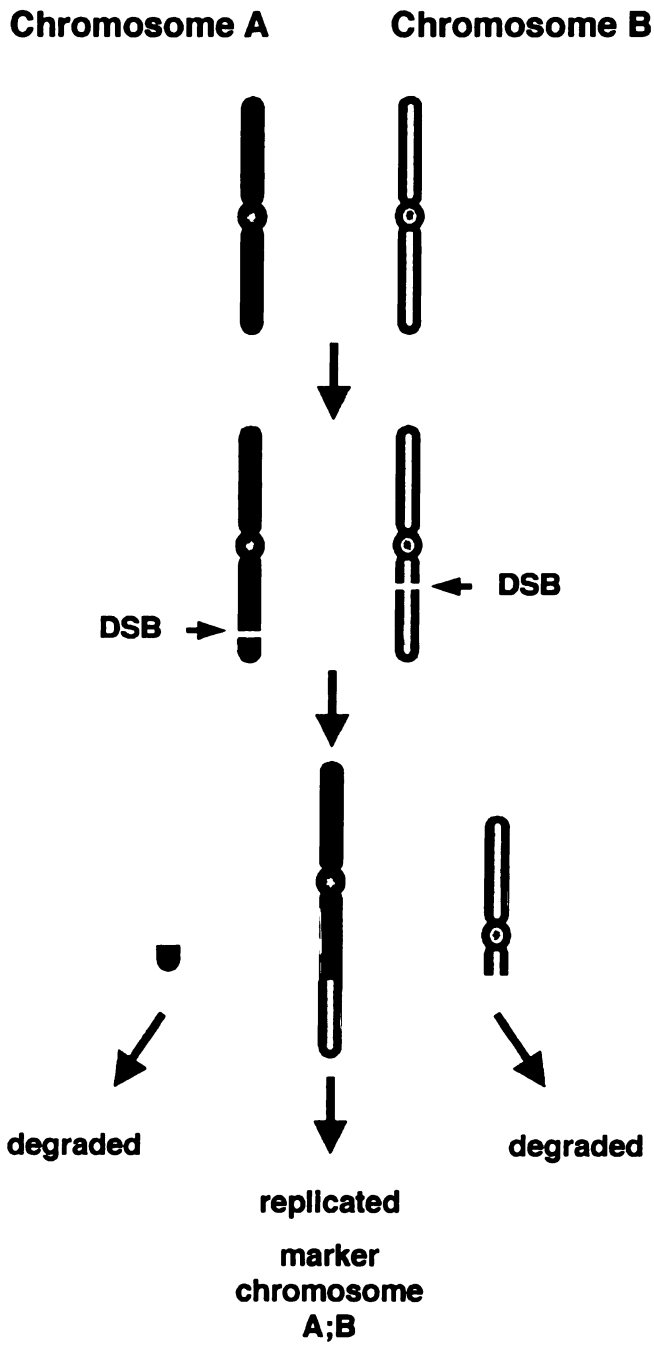

Figure 6. Diagram of marker chromosomes found in MSU-1.1 cells (36).

(12q11.2→12pter), and 15 (15p11.2→15pter), and a partial trisomy of chromosome 1 (1qter→1p13). Additional details regarding these marker chromosomes are discussed below. The two marker chromosomes are unique to these cells and they have been stably maintained through hundreds of population doublings. Studies have shown that MSU-1.1 cells have normal expression of the two common TSGs, *p53* (273, 274), and *Rb/p105* (McCormick *et al.*, unpublished studies), although it is not known whether downstream effector pathways of *Rb/p105* are altered. The LG1, MSU-1.0 and MSU-1.1 cells have a spindle-shaped morphology, do not form foci *in vitro*, and do not form tumors in athymic mice (McCormick *et al.*, unpublished studies).

One critical difference between MSU-1.0 and MSU-1.1 cells is that MSU-1.1 cells can be malignantly transformed by overexpression of a transfected *H-RAS* (38), *v-K-RAS* (275), *N-RAS* (37), or *v-SIS* (276) oncogene, or expression of the *v-FES* oncogene in combination with expression of the *H-RAS* oncogene (169) and suitable selection. In addition, MSU-1.1 cells have also been transformed into malignant cells by a single exposure to carcinogenic agents, such as an active derivative of benzo(a)pyrene (277), *N*-Methyl-*N*-nitrosourea (278), ethyl nitrosourea (McCormick *et al.*, unpublished studies), ultraviolet radiation (279), and ionizing radiation (273, 274) and suitable selection. The carcinogen transformed cells were identified by their ability to form distinct foci on a monolayer of non-transformed cells. The MSU-1.0 cells, from which the MSU-1.1 cells were derived, have never been transformed into tumor-forming cells,

despite overexpression of the *H-RAS* oncogene, carcinogen-treatment, or the use of combinations of such treatments and suitable selection (McCormick *et al.*, unpublished studies) (280). Similar results were reported by Hahn *et al.*, 1999 (168) for telomerase expressing human fibroblasts that were transfected with the *H-RAS* oncogene. These findings suggest that the loss and/or gain of genetic material that resulted in the formation of the two marker chromosomes plays a critical role in the ability of MSU-1.1 cells to be malignantly transformed. Unlike the carcinogen-treated, malignantly transformed MSU-1.1 cells that are *p53* mutant (273, 274, 278), the oncogene-transfected malignantly transformed MSU-1.1 cells have normal functioning *p53* (McCormick *et al.*, unpublished studies), and are chromosomally stable (38, 275).

The two marker chromosomes found in MSU-1.1 cells (Figures 5 and 6) are the result of two independent interchromosomal translocations that occurred as a result of DSBs to the four chromosomes involved. Marker chromosome 1 (M1) is made up of the q arm terminus to 1p13 of chromosome 1 and most of chromosome 11; t(1;11)(1qter→1p13 : 11p15→11qter) (274). The missing region of chromosome 11 is 11p15 to the p arm terminus. This trisomeric region of chromosome 1 is likely the result of a nondisjunctional event that occurred during mitosis, resulting in a trisomy of chromosome 1, followed by a subsequent DSB and formation of M1. Marker chromosome 2 (M2) is made up of a translocation between chromosome 12 and chromosome 15, in which the entire p arm and a small portion of the q arm of chromosome 12 and half of the p arm

of chromosome 15 have been lost; t(12;15)(12qter→12q11.2 : 15p11.2→15qter) (274). The missing regions involved in this marker chromosome are 12q11.2 to the p arm terminus of chromosome 12 and 15p11.2 to the p arm terminus of chromosome 15. These genetic alterations might provide a means of TSG LOH through the loss of DNA from chromosomes 11, 12, and/or 15, or oncogene activation as a result of the partial triploidy of chromosome 1 or the movement of a gene to a new promoter as a result of one of the rearrangements.

a. Mechanisms of Formation of Marker Chromosomes

The formation of marker chromosomes, such as those found in MSU-1.1 cells, require DSBs that occur as a result of ionizing radiation damage by cosmic rays or, more commonly, by spontaneous breaks such as those that occur during DNA replication or those produced by reactive oxygen species (281). In normal mammalian cells, the estimated rate of DSB formation by endogenous processes is approximately 4.33×10^{-3} DSB/cell/hour (282). Most DSBs are repaired, and are thus not observed. Inadequate or slow repair of a single DSB can result in degradation of DNA located at the breakpoint, and the loss of genes located in those regions (283, 284). When a second DSB occurs on another chromosome before the first DSB is repaired, it is possible that chromosomal translocations can occur, resulting in the formation of a marker chromosome(s) (Figure 7) (283, 284). It has been demonstrated in mouse embryonic stem cells, *in vitro*, that when two DSBs are simultaneously present in these cells, translocations can

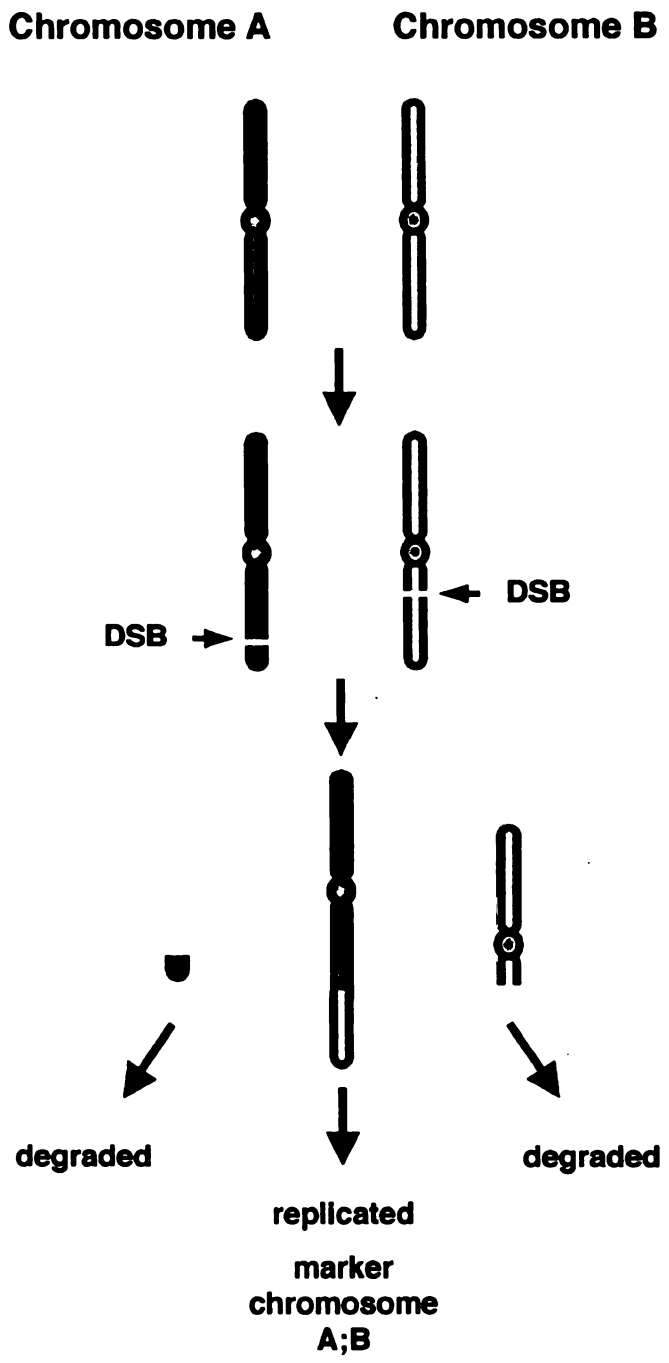

Figure 7. A schematic diagram of the occurrence of DSBs and the formation of a marker chromosome.

occur at a frequency of 1 x 10^{-4}/viable cell (285).

In human cells, repair of DSBs occurs mainly by nonhomologous end-joining (NHEJ) (286, 287). NHEJ involves the joining and ligation of two nonhomologous chromosome ends created by DSBs (286-289). Although the exact mechanism involved is not known, DNA-dependent protein kinase (DNA-PK) is a key protein involved in this repair process (290, 291). DNA-PK is a nuclear serine-threonine multi-subunit protein kinase that is activated upon DNA binding (292). The initiation of repair occurs by recognition of the DSB by the DNA-PK subunits Ku70 and Ku80. Ku70 and Ku80 then activate the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) by stabilizing its interaction with the DNA ends. DNA-PKcs then phosphorylates and activates other proteins necessary for the repair process, including DNA polymerases, DNA ligase IV (292), and XRCC4, a protein involved in stabilizing DNA ligase IV (293).

DSBs that occur in non-telomeric regions of the chromosome produce new chromosomal ends that are unprotected by telomeres, and are therefore susceptible to exonuclease degradation (294, 295). Telomeres, as discussed above, are DNA regions located on the ends of normal human chromosomes that function to protect and stabilize the chromosome (218). The joining of one chromosome to another by NHEJ acts as a protective measure by producing a "new" chromosome that now contains a telomere at each end. For such a "new"

chromosome to be stably inherited, it must have a single intact centromere which is required for mitotic spindle attachment and separation during mitosis.

b. Role of Marker Chromosomes in Cancer

The genetic alterations that occur from the formation of marker chromosomes in carcinogenesis can result in the activation of proto-oncogenes by placing such a gene under control of a different promoter or they may result in a novel oncogenic fusion gene (283, 296). The genetic alterations may also inactivate one allele of a TSG (283, 296). The presence and/or increase in occurrence of marker chromosomes are correlated with an increase in the risk of cancer (297). Although the formation of marker chromosomes occurs randomly, specific marker chromosomes are characteristic of particular types of cancer, such as those found in chronic myeloid leukemia (CML). The reciprocal translocation between chromosome 9 and chromosome 22 that occurs in CML results in the "Philadelphia chromosome" (so-called because of its joint-discovery at two institutions in Philadelphia in 1960) (298). This reciprocal translocation produces a fusion gene with the *c-ABL* gene from chromosome 9 to the *BCR* gene from chromosome 22 (the Philadelphia chromosome) and the *c-SIS* proto-oncogene being translocated from the q arm of chromosome 22 to chromosome 9 (298). The c-Abl protein is a non-receptor tyrosine kinase involved in many cellular functions including Myc protein expression, resulting in an increase in cell proliferation, activation of the anti-apoptotic proteins Bcl-2 and Akt, and integrin alterations, which decrease cell adhesion (298). The Bcr protein is a serine-

threonine kinase that can also function as a GDP/GTP exchange factor (298). Bcr activates the Ras-Raf-1-MEK-ERK signal transduction pathway by activating Grb2 and by enhancing Ras activity by increasing the conversion of GDP to GTP. The fusion protein that results from the *BCR-ABL* gene retains all of the transforming functions of each protein (299). It has been shown that the Bcr region contains an oligomerization domain that promotes homodimerization of the Bcr-Abl protein, resulting in autophosphorylation and activation of the tyrosine kinase function of Abl (299).

Another example of the role of marker chromosomes in cancer is the reciprocal translocation between chromosome 8 and chromosome 2, 4, or 22 that occurs in Burkett's lymphoma (184). These translocations result in the fusion of the *c-MYC* gene (located at 8q24) with a constitutively active immunoglobulin (*IG*) gene (*IGK* at 2p12, *IGH* at 14q32, or *IGL* at 22q11) (184). This translocation results in continuous expression of the c-Myc protein without transcriptional down-regulation.

Although the discovery of specific chromosome markers associated with specific cancers has led to the identification of oncogenes (or oncogenic fusion genes), the formation of marker chromosomes in cancer may also indicate that DNA loss has occurred. As noted above, inadequate or slow repair of DSBs can result in DNA degradation (283, 284), which might contribute to LOH of a TSG (45, 46). In fact, detectable regions of chromosomal DNA loss has led to the identification of many TSGs associated with various cancers, such as *p53* (31)

and *PTEN* (300, 301). The *PTEN* protein product is a phosphatase that is associated with the regulation of many normal cellular processes, including growth, adhesion, migration, invasion and apoptosis (302, 303). In addition, loss of DNA as a result of the formation of marker chromosomes in various cancers has identified these deleted regions as having tumor suppressor ability (304-306).

c. Speculation of the Relevance of the Marker Chromosomes in the Malignant Transformability of MSU-1.1 Cells

As noted above, MSU-1.1 cells have a growth advantage over their parental MSU-1.0 cells (36). The genetic alterations involved in the formation of one or both of the marker chromosomes might play a causal role in this growth advantage. Specifically, the genetic alterations might provide a means of TSG LOH because of the loss of DNA on chromosomes 11, 12, and/or 15, or oncogene activation as a result of the partial trisomy of chromosome 1 or chromosomal rearrangement.

A common critical event in the multi-step process of carcinogenesis is an increase in cellular growth rate following a genetic or epigenetic alteration. If the genetic alteration involved in the formation of the first marker chromosome provided a growth advantage for that cell, such that upon clonal expansion, the majority of the cells in the population now contain that marker chromosome, the probability of one of those cells obtaining two additional DSBs in order to form

the second marker chromosome would be much greater. If the acquisition of the second marker chromosome provided a further growth advantage, these cells would gain in frequency in a population of cells in culture. If the first or second marker chromosome did not provide a growth advantage for the cell that had this change, the probability of that cell being detected in a large population of cells in culture would be extremely small. It is also possible that the marker chromosomes might have been formed simultaneously with both, or more likely only one, providing the growth advantage. Unfortunately, there were no intermediate cell types detected (i.e. MSU-1.0 cells with only one marker) in the derivation of MSU-1.1 cells; therefore it is not known whether the growth advantage occurred due to the sequential or simultaneous formation of the two marker chromosomes.

The growth advantage observed in MSU-1.1 cells might be the result of the activation of a proto-oncogene or the creation of a novel fusion oncogene. However, there is no evidence in the literature demonstrating the existence of either type of oncogene located on the chromosomal regions involved in the formation of the two marker chromosomes in MSU-1.1 cells. Alternatively the growth advantage might be the result of the loss of TSG function. Evidence in the literature implicates the regions of chromosome 11 (307-309) and chromosome 12 (310), that are lost in MSU-1.1 cells, as possessing tumor suppressor ability in other tumor-derived cell strains. Although the loss of DNA to chromosome 15 in MSU-1.1 cells has been shown to occur in various cancer

types, the relevance of this region in the carcinogenesis process is not known. A recent review of the occurrence of chromosome arm loss in seventy-three different human tumor types showed that losses of 11p, 12p, and 15p occurred in 19%, 5%, and 0% of tumors, respectively (311). A small percentage of those tumor types in which 11p was identified to be missing occurred in sarcomas. The putative roles of the lost chromosome fragments and the partial trisomy of chromosome 1 in carcinogenesis (discussed in greater detail below) suggests that the missing region of chromosome 11 in MSU-1.1 cells (11p15.5→11pter) is most likely to harbor a TSG(s) that is involved in the malignant transformation of MSU-1.1 cells.

As noted above, for most TSGs, both alleles must lose the ability to code for functional protein for loss of gene function to occur. Thus, I postulate that the other allele of the affected TSG in MSU-1.1 cells has lost the ability to make functional protein by mutation or an epigenetic modification, or that one functional allele does not result in a sufficient amount of the TSG protein (i.e. haplo-insufficiency). In addition, based on the occurrence and relevance of DSBs and the formation of marker chromosomes as noted above, it would appear that one chromosome of each marker found in MSU-1.1 cells serves to protect the other chromosome by providing a telomere, thereby preventing further exonuclease degradation of the other chromosome. Specifically, based on the roles that these chromosomes play in carcinogenesis (as noted above and detailed below), I postulate that the partial trisomy of chromosome 1 is acting as the protector for

chromosome 11 in Marker 1, and that chromosome 15 is acting as the protector for chromosome 12 in Marker 2. As noted above, the DNA fragments lost in the formation of these marker chromosomes might account for LOH of a TSG located in these regions. However, based on statistical considerations, one would not expect a TSG to be located on each of the three DNA fragments lost in MSU-1.1 cells. It is more likely that a TSG is located on the missing DNA fragment of 11p (Marker 1) and/or another TSG is located on the 12p lost DNA fragment (Marker 2). The relevance of each of the genetic alterations in carcinogenesis is detailed below.

i. Relevance of Chromosome 11p in Carcinogenesis

Several studies have shown that there is frequent LOH on chromosome 11p and that this arm harbors many potential TSGs. Specifically, LOH of the region 11p15 (Marker 1) has been studied extensively and has been shown to occur in Wilms' tumor (a childhood form of kidney cancer) (312), adrenocortical carcinoma, hepatoblastoma, bladder carcinoma, testicular carcinoma, ovarian carcinoma, and breast cancer (50). Of particular interest is the LOH of 11p15 that occurs in rhabdomyosarcomas, a skeletal muscle sarcoma that is closely related to fibrosarcomas (50, 306, 311, 313). These findings suggest that loss of expression of a TSG(s) located in the region of 11p15 may be involved in the development of many tumor types, and more specifically, soft tissue sarcomas. Using microcell mediated chromosome transfer (MMCT) of chromosome 11 into

a wide variety of tumor-derived cell lines (including fibrosarcomas), functional studies have shown that this chromosome inhibits tumor formation (305, 307-309, 314-319). The study by Dowdy *et al.*, 1990 (317), was one of the first to indicate that a TSG is located in the p15 region of chromosome 11 and is causally involved in Wilms' tumor. Since these studies, many candidate TSGs have been mapped to the 11p15 region in a variety of cancers (306, 320-328), and some of these TSGs have been identified. These include *TSG101* (a TSG associated with breast cancer) (329), *ELF5* (expresses an ETS transcription factor involved in regulating cell proliferation) (330), *HTS1* or *ST5* (thought to be involved in a cell growth regulatory pathway) (331, 332), *WEE1^{Hu}* (expresses a Cdc2 inhibitory kinase involved in cell cycle progression) (333), and *p57^{KIP2}* (a member of the p21 family that inhibits Cdks involved in cell cycle progression) (334).

A major problem with attempting to identify genes located on the 11p15 region is its large size (10 Mb) (322). To identify TSGs located on this region, high-resolution physical maps have been constructed (322, 335-339). One method that has been employed to localize the region in which a possible TSG might be located is irradiation microcell mediated chromosome transfer (XMMCT). This method involves the use of gamma radiation, which produces random chromosomal deletions. This method has been used to localize the region of 11p15 as harboring several TSGs (313, 317, 340).

Studies have shown that 11p15 contains regions of frequent LOH (50). It has also been recently shown that 11p15 contains many transposable elements, which result in the frequent occurrence of DNA DSBs in this region (322). This region also contains several growth related genes in which genomic imprinting and epigenetic silencing, due to DNA methylation, occur (306, 327, 341-343).

II. Relevance of Chromosome 1 in Carcinogenesis

There is limited evidence suggesting a role for the trisomeric q arm of chromosome 1 in carcinogenesis. Relevant to this dissertation, a gain (trisomy) of chromosome 1 has occurred in a few cases of osteosarcomas (344). Osteosarcomas are a member of the sarcoma family of tumors, which also include fibrosarcomas. Two studies involving MMCT of chromosome 1 into HT1080 human fibrosarcoma cells have shown contradictory results. The first study showed that MMCT of this chromosome resulted in suppression of tumorigenicity (319). However, a later study showed that introduction of this chromosome had no effect on the tumorigenicity of HT1080 cells (345). These authors showed that HT1080 cells that now had trisomy of chromosome 1 also showed no differences in morphology and growth properties *in vitro* when compared with the parental HT1080 cells, suggesting that the trisomy of chromosome 1 does not play a role in carcinogenesis (345). In addition, the authors suggested that the contrast between their results and that of the previous

study (319) might be because a "less malignant variant" of the HT1080 cell strain that was used in the earlier study.

iii. Relevance of Chromosome 12p in Carcinogenesis

Studies have shown that the loss of the p arm of chromosome 12 (lost in Marker 2 of MSU-1.1) is involved in ovarian cancer (346), acute lymphoblastic leukemia (347), lung cancer (348), and prostate cancer (349). One functional study has been performed involving MMCT of chromosome 12 (310). The investigators showed that the introduction of the p arm of chromosome 12 into a human prostate cancer cell line suppressed the tumorigenicity of these cells. These findings suggest a candidate TSG(s) is located on chromosome 12p. While some studies have shown chromosome 12 loss in cancer, others have shown that gain of chromosome 12p (trisomy) is involved in adenocarcinomas (350) and is also a common occurrence in testicular germ cell tumors (351-353). Introduction of chromosome 12 into a human lung adenocarcinoma cell line did not suppress tumorigenicity (316).

iv. Relevance of Chromosome 15 in Carcinogenesis

There is little evidence implicating chromosome 15 in human cancer, but some studies have shown that the loss of this chromosome might be involved in a few cases of human skin (354), breast (355), and head and neck (356) carcinomas. The limited results provided from these studies suggest that the

loss of chromosome 15 is involved in tumor progression and malignancy. Introduction of this chromosome into a human skin carcinoma cell line suppressed tumor growth (357). These investigators identified the gene involved in tumor suppression as *THBS1*. *THBS1* is located at 15q15 (358) and has been shown to inhibit tumor angiogenesis and metastasis (62). There are no reports of any genes located on the p arm of chromosome 15 and no results indicating the relevance of the loss of the p arm of this chromosome in cancer.

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CHAPTER II

INTRODUCTION OF CHROMOSOME 15 INTO THE IMMORTALIZED HUMAN FIBROBLAST CELL STRAIN MSU-1.1 PREVENTS MALIGNANT TRANSFORMATION BY THE T24 *H-RAS* ONCOGENE

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The abbreviations used are:

FBS, fetal bovine serum; FISH, fluorescent *in situ* hybridization; MEM, modified Eagle's medium; MMCT, microcell mediated chromosome transfer; SCS, supplemented calf serum; SDS, Sodium dodecyl sulphate; TBST, Tris-buffered saline with tween; TG^R, thioguanine-resistant; THBS1, Thrombospondin.

ABSTRACT

Although diploid mortal and immortal human skin fibroblasts cannot be transformed into malignant cells by T24 *H-RAS* oncogene overexpression, a cell strain derived from diploid immortal human fibroblasts (MSU-1.1) was readily transformable into malignant cells by overexpression of the T24 *H-RAS* oncogene (Hurlin *et al.*, 1989). MSU-1.1 cells differ from diploid mortal and immortal human fibroblasts in that they exhibit a loss of DNA fragments on chromosomes 11 (11p15→11pter), 12 (12q11.2→12pter), and 15 (15p11.2→15pter) and possess a partial trisomy of chromosome 1 (1qter→1p13). These genetic alterations suggest that one or more of the lost DNA fragments may be required for the malignant transformation of these cells by the T24 *H-RAS* oncogene. To test this hypothesis, we transferred a normal human 11, 12, or 15 chromosome into an MSU-1.1 cell strain (MSU-1.1 TG^R). The resultant chromosome hybrid clonal populations were assessed for malignant transformability by transfection with the T24 *H-RAS* oncogene. The results indicate that the introduction of chromosome 15, but not chromosomes 11 or 12, prevented the malignant transformation by T24 *H-RAS* oncogene overexpression. The T24 *H-RAS* transformed MSU-1.1 chromosome 15 hybrid clonal populations had *in vitro* properties similar to those of the control T24 *H-RAS* transformed MSU-1.1 cell strain; both exhibited a transformed morphology, similar growth rates, and the ability to form colonies in agarose. However, the control T24 *H-RAS* transformed MSU-1.1 cell strain, the hybrid clonal populations

did not form tumors when injected into athymic mice. The introduction of chromosome 11 or 12 had no effect on T24 *H-RAS* transformation of MSU-1.1 cells into malignant cells. To verify these findings, we transferred a normal human 11, 12, or 15 chromosome into an MSU-1.1 T24 *H-RAS* transformed tumor-derived cell strain, PH2MT, which is karyotypically identical to the MSU-1.1 cell strain. Just as with the MSU-1.1 cells, the introduction of chromosome 15 into PH2MT cells resulted in clonal populations that were not tumorigenic or were only slightly tumorigenic. In addition, a majority of the non-tumorigenic hybrid clonal populations lost the characteristics of the T24 *H-RAS* transformed cells. These cells now had a spindle-shaped morphology, grew at a slower rate, and did not form colonies in agarose. The introduction of chromosome 11 or 12 into the PH2MT cell strain had no effect on morphology, growth rate, ability to form colonies in agarose, or tumorigenicity of the resultant hybrid clonal populations. These novel findings suggest tumor suppressor activity localized to chromosome 15. This is the first demonstration that the introduction of chromosome 15 into either immortal or tumorigenic cell strains can suppress or reverse the tumorigenicity state of the recipient cell strain.

INTRODUCTION

To provide an *in vitro* model for the malignant transformation of human fibroblasts, McCormick and his colleagues developed the MSU-1 lineage of human fibroblasts (1). They derived a fibroblast cell line designated LG1 from foreskin tissue of a normal neonate. From the LG1 cells, they clonally derived a diploid, infinite lifespan, telomerase positive, cell strain designated MSU-1.0 (1, 2). From the MSU-1.0 cells, they isolated a spontaneous, more rapidly growing variant cell strain designated MSU-1.1 (1, 2). The MSU-1.1 cells have a stable complement of 45 chromosomes, including two marker chromosomes. As a result of the formation of the marker chromosomes, MSU-1.1 cells exhibit a loss of DNA fragments and a partial trisomy of chromosome 1 (see below). The LG1, MSU-1.0 and MSU-1.1 cells have a normal spindle-shaped morphology, do not form foci *in vitro*, and do not form tumors in athymic mice. MSU-1.1 cells can be malignantly transformed by overexpression of a transfected *H-RAS* (3), *v-K-RAS* (4), or *N-RAS* (5) oncogene, expression of the *v-SIS* oncogene (6), or expression of the *v-FES* oncogene in combination with expression of the *H-RAS* oncogene (7) and suitable selection. In addition, MSU-1.1 cells have also been transformed into malignant cells by a single exposure to carcinogenic agents, such as an active derivative of benzo(a)pyrene (8), *N*-Methyl-*N*-nitrosourea (9), ethyl nitrosourea (McCormick *et al.*, unpublished studies), ultraviolet radiation (10), and ionizing radiation (11, 12) and suitable selection. The MSU-1.0 cell strain, from which the MSU-1.1 cells were derived, has never been transformed

into tumor-forming cells; despite overexpression of the *H-RAS* oncogene, carcinogen-treatment, or the use of combinations of such treatments and suitable selection (McCormick *et al.*, unpublished studies) (8). These results are consistent with the recent findings of Hahn *et al.*, 1999 (13), who found that human fibroblasts that gain an infinite lifespan by expression of telomerase could not be transformed to malignancy by overexpression of the *H-RAS* oncogene. These findings suggest that one or more of the genetic alterations involved in the formation of the two unique marker chromosomes allow the MSU-1.1 cells to be malignantly transformed.

Marker chromosome 1 (M1) of the MSU-1.1 cells is the result of a translocation involving chromosome 11 and a trisomeric fragment of chromosome 1 (t[1;11][1qter→1p13 : 11p15→11qter]) (1, 12). Marker chromosome 2 (M2) is the result of a translocation involving chromosomes 12 and 15 (t[12;15][12qter→12q11.2 : 15p11.2→15qter]) (1, 12). The two marker chromosomes are unique to this cell strain and they have been stably maintained through hundreds of population doublings.

The analysis of marker chromosomes in particular types of human tumors has led to the identification of genes involved in the carcinogenesis process (14-16). When a chromosome or a fragment of a chromosome has been lost in cancer cells, one approach to determining whether this loss contributes to the transformed properties of the cells is to return the lost chromosome to the cell. This can be done by microcell mediated chromosome transfer (MMCT). If cells

carrying the transferred chromosome lose the ability to form tumors or lose some other transformation-related characteristic, it may indicate that the lost DNA played a causal role in malignant transformation. The transfer of specific chromosomes into various human tumor-derived cell lines has been shown to result in the suppression of the ability of these cells to form tumors (17-22). In some cases, the tumor suppressor activity has been localized to a discrete region of a chromosome (17, 23-26) or a specific tumor suppressor gene (TSG) (27-32).

Typical studies using the MMCT technique involve returning a normal human chromosome to cancer cells that have lost a fragment of the homologous chromosome (33). This makes the cell diploid for the region of the lost chromosomal fragment, but triploid for the rest of that chromosome. If the cells carrying the introduced chromosome lose the ability to form tumors or lose some other transformation-related characteristic, it suggests that a TSG is located on the lost DNA fragment and that it plays a causal role in malignant transformation. However, it is also possible that a gene(s) on the chromosomal region that is now triploid might also play a causal role.

To determine whether the DNA fragments lost in the formation of chromosomal markers M1 and M2 might play a role in the transformation of MSU-1.1 cells, we carefully reviewed the literature. There was strong evidence implicating the loss of the 11p15→11pter region in Wilms' tumor (34), adrenocortical carcinoma, hepatoblastoma, bladder carcinoma, testicular

carcinoma, ovarian carcinoma, and breast cancer (35). Several studies involving MMCT of chromosome 11 have been performed in various human tumor-derived cell lines, demonstrating the tumor suppression ability of chromosome 11 (18-22, 26, 36-39). These studies have led to the identification of several genes in the 11p15→11pter region with possible roles associated with tumor suppression (30, 40-48). The region also contains several growth-related genes in which genomic imprinting and epigenetic silencing by DNA methylation occur (29, 49-52).

There was also evidence that the loss of the p arm of chromosome 12 plays a causal role in childhood leukemia (53, 54), prostate cancer (55), and ovarian cancer (56). Introduction of the p arm of chromosome 12 into a human prostate cancer cell line resulted in cells that did not form tumors in athymic mice, suggesting the presence of a TSG on 12p (25). One putative TSG identified in the 12p13 region of chromosome 12 is $p27^{KIP1}$, which is lost in many cancers (57). There were no reports indicating that the loss of the p arm of chromosome 15 is correlated with carcinogenesis. In fact, there are no known genes located on the p arm of this chromosome.

As noted above, MSU-1.1 cells also have a gain of chromosomal material as a result of the partial trisomy of chromosome 1 (1qter→1p13). It is possible that this region of chromosome 1 might contain an oncogene. Because of this trisomy, one would expect that there are three copies of such a gene instead of two; however the literature does not indicate a role for this region in carcinogenesis.

Because MSU-1.1 cells, unlike their diploid precursor MSU-1.0 cells, can be transformed to malignant cells by various methods, we hypothesize that the loss of chromosome fragments on chromosomes 11, 12, and/or 15 play a causal role in this transformability. To test this hypothesis, MMCT was performed to introduce each chromosome individually into the MSU-1.1 thioguanine resistant derivative cell strain, MSU-1.1 TG^R (58), and the tumor derived MSU-1.1 T24 *H-RAS* malignantly transformed cell strain, PH2MT (3). The introduction of chromosome 15 into the MSU-1.1 TG^R cell strain, but not of chromosome 11 or 12, prevented the malignant transformation by the T24 *H-RAS* oncogene in four out of five hybrid clones examined. In support of these findings, the introduction of chromosome 15 into the PH2MT cell strain, but not of chromosome 11 or 12, inhibited or suppressed tumorigenicity in four out of six hybrid clones examined. Collectively, the findings presented here provide evidence for tumor suppressor activity localized to chromosome 15.

MATERIALS AND METHODS

Cell Culture. All cells were cultured in modified Eagle's medium containing L-aspartic acid (0.2 mM), L-serine (0.2 mM) and pyruvate (1 mM) (MEM), supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and hydrocortisone (1 μg/ml) (designated complete MEM). The MSU-1.1 (1), MSU-1.1 TG^R (58), and PH2MT (3) cell strains, were grown in complete MEM with 10% supplemented calf serum (SCS) (Hyclone, Logan, UT).

The chromosome donor mouse A9 somatic hybrid cell strains carrying human chromosome 11, 12, or 15 (59), were grown in complete MEM supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 400 units/ml hygromycin B (Calbiochem, San Diego, CA). All cells were maintained in a humidified incubator with 5% CO_2 at 37 °C.

Microcell Mediated Chromosome Transfer (MMCT). MSU-1.1 TG^R and PH2MT derived hybrid clonal populations were generated using chromosome donor mouse A9 somatic hybrid cell strains that contained either human chromosome 11, 12, or 15; each marked with the gene for hygromycin B resistance (59). MMCT was performed as described previously (59). Briefly, chromosome donor cell strains were plated in 25-cm^2 NUNC flasks (Nalge Nunc International, Denmark) at a density of 1 x 10^6 cells per flask. After 24 h, the medium was aspirated off and complete MEM plus 10% FBS and 0.2 μg/ml colcemid (Sigma-Aldrich, St. Louis, MO) was added. The medium was kept on

the cells for 48 h to induce metaphase arrest and micronucleation. Enucleation of the micronuclei was carried out by centrifugating the cells in the presence of MEM containing 10 μg/ml cytochalasin B (Sigma-Aldrich, St. Louis, MO) at 9,500 x g for 1 h at 35 °C. The resultant microcell pellets were isolated and re-suspended in MEM. This microcell suspension was filtered through 8 μm, and subsequently, 5 μm polycarbonate filters (Nucleopore Corp., Pleasanton, CA). The purified microcells were allowed to attach to the recipient MSU-1.1 TG^R or PH2MT cells (plated 24 h earlier at a density of 0.75 x 10^6 cells per 25-cm^2 flask) using MEM containing 100 μg/ml phytohemagglutinin-P (Sigma-Aldrich, St. Louis, MO). After 25 min, the medium was aspirated off the cells and MEM containing 50% polyethylene glycol (mw 1,450) (Sigma-Aldrich, St. Louis, MO) was added in order to induce fusion of the microcells to the recipient cells. After 1 min, the medium was aspirated off, and complete MEM supplemented with 10% SCS was added. After 24 h, the cells were trypsinized and plated into ten 100-mm diameter dishes with complete MEM supplemented with 10% SCS and 125 units/ml hygromycin B. Culture dishes received fresh complete MEM supplemented with 10% SCS and 125 units/ml hygromycin B weekly. After 3 weeks, hygromycin B-resistant clonal populations were visible and they were isolated. All chromosome hybrid clonal populations were cultured and maintained in the presence of 125 units/ml hygromycin B.

Fluorescent *In Situ* Hybridization (FISH). FISH analysis was performed on chromosome metaphase spreads and interphase cell spreads. Spreads were obtained by subjecting the cells to complete MEM supplemented with 10% FBS and colcemid (10 μg/ml for recipient cell strains and hybrid clonal populations or 1 μg/ml for A9 chromosome donor cell strains). After 3 h, the medium was removed and the cells were re-suspended in 0.075M KCl hypotonic solution and incubated at 37 °C for 30 min. The cells were pelleted, re-suspended in methanol/acetic acid (3:1, vol/vol) fixative, and then dropped onto glass slides and air-dried.

For chromosomes 11 and 12, human chromosome painting probes (Roche Molecular Biochemicals, Indianapolis, IN) and Whole Chromosome Paint DNA FISH probes (Vysis, Inc., Downers Grove, IL) were used on chromosome metaphase spreads. For chromosome 15, Chromosome Enumeration Paint DNA FISH probes (Vysis, Inc., Downers Grove, IL) were used on interphase cell spreads. Slides were counterstained with 1.5 μg/ml 4',6-diamidino-2-phenylindole in Vectashield anti-fade mounting medium (Vector Laboratories, Burlingame, CA) and visualized using fluorescence microscopy. Images in this thesis/dissertation are presented in color.

T24 *H-RAS* Plasmid Construction and Transfection. The pcDNA6/T24 *H-RAS* vector was constructed in this laboratory by isolating the 6.6 kb *BamHI* fragment containing the T24 *H-RAS* oncogene (60) from the vector pHO6T1 (61),

and inserting it into the *BamHI* cloning site of pcDNA6/V5-His (Invitrogen, Carlsbad, CA). This vector contains the blasticidin resistance gene.

Transfection was performed using LipofectAMINE according to the transfection protocol provided by the supplier (Invitrogen Corp., Carlsbad, CA). $6x10^5$ cells per 100-mm diameter dish were transfected with 3 μg pcDNA6/T24 *H-RAS* DNA. Clones were selected in complete MEM containing 10% SCS and 2.5 μg/ml blasticidin S (Invitrogen Corp., Carlsbad, CA). Culture dishes received fresh complete MEM supplemented with 10% SCS and 2.5 μg/ml blasticidin S weekly, and after three weeks, clones were isolated.

Western Blot Analysis. Western blot analysis was performed as previously described (62). Briefly, whole cell protein preparations were prepared from log-phase cells. Protein samples (25 μg) were denatured at 95 °C for 10 min, resolved on a 10% SDS-polyacrylamide gel via electrophoresis, and transferred onto a nitrocellulose membrane. After transfer, the membrane was blocked for 2 h with 5% milk/TBST and then incubated with rabbit anti-H-Ras (C-20) polyclonal antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-β actin (clone AC-15) monoclonal antibody (1:25000; Sigma-Aldrich, St. Louis, MO) for 2 h. The membrane was washed three times with TBST and then incubated with horseradish peroxidase conjugated goat anti-rabbit (1:5000; Sigma, St. Louis, MO) or goat anti-mouse (1:5000; Sigma-Aldrich, St. Louis, MO) antibodies for 1 h. The membrane was washed again three times with TBST.

Antibody binding was detected using ECL detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to x-ray film.

Assay for Tumorigenicity. Athymic male and female BALB/c mice 4-6 weeks of age were injected subcutaneously into the right and left rear flanks with 1 x 10^6 cells suspended in 0.2 ml of MEM, per site. The mice were monitored weekly for tumor growth, and the size of each tumor was measured using a caliper. When the tumor reached approximately 1 cm in diameter the mice were sacrificed, and the tumors removed. Tumor volume was calculated using the formula for the volume of a hemi-ellipsoid; Volume = 0.5236 x length x width x height. All tumors were histologically verified. Cells obtained from a maximum of two tumors that had formed for each hybrid clonal population were returned to culture and maintained in the presence of complete MEM supplemented with 10% SCS and 150 units/ml hygromycin B for future analysis. All mice were sacrificed six months after injection, and any tumors present were removed. A minimum of two mice (four injection sites) were used for each cell strain tested.

Assay for Ability to Grow in Agarose. Cells were assayed for ability to form colonies in 0.33% agarose (SeaPlaque) (FMC Bioproducts, Rockland ME). A total of 5 x 10^3 cells per 60-mm diameter plate were plated in triplicate in 1.5 ml of top 0.33% agarose with complete MEM supplemented with 2% FBS. The top layer was poured over a 5 ml 2% agarose/complete MEM supplemented with 2%

FBS bottom layer. The plates were placed in a humidified incubator with 3% CO_2 at 37 °C. After 24 h, 3.5 ml complete MEM supplemented with 2% FBS and 20mM HEPES was placed over the agarose. This medium was replaced weekly with 3.5 ml fresh complete MEM supplemented with 2% FBS. After 22 days, the medium was removed, and the cells in the agarose were fixed in 3 ml 2.5% glutaraldehyde. Three representative fields were digitally captured from each 60-mm diameter plate and analyzed for colony size (diameter) using NIH Image 1.6.2 (National Institutes of Health, Bethesda, MD).

Determination of *In Vitro* Growth Rate. Cells were assayed for their growth rate in complete MEM supplemented with 10% SCS. A total of 7.5×10^4 cells per 60-mm diameter plate were plated into a sufficient number of plates to allow the cells to be counted in duplicate every 24 h for a minimum of 6 days. The medium on the cells was replaced with complete MEM supplemented with 10% SCS on Day 3. The growth rate for each hybrid clonal population was determined from the exponential growth phase of each growth curve.

RESULTS

Chromosome introduction into MSU-1.1 TG^R cells and verification of hybrid clonal populations. To determine if the loss of genetic material from MSU-1.1 cells as a result of the formation of the two marker chromosomes was required for the malignant transformation by the T24 *H-RAS* oncogene, we introduced one copy of chromosome 11, 12, or 15 into the MSU-1.1 TG^R cell strain by microcell mediated chromosome transfer (MMCT). Several independent, hygromycin B-resistant hybrid clonal populations for each transferred chromosome were isolated from two independent chromosome transfer experiments. Ten drug resistant hybrid clonal populations were obtained from each chromosome transfer. Hybrid clonal populations were verified for the presence of the appropriate extra chromosome by fluorescent *in situ* hybridization (FISH) analysis using chromosome specific probes. A representative hybrid clonal population for each chromosome group is shown in Figure 1. All hybrid clonal populations examined by FISH analysis were positive for the presence of the appropriate extra chromosome and a minimum of two independent verified hybrid clonal populations for each chromosome group were used for further study. FISH analysis was also performed for each chromosome donor mouse A9 somatic hybrid cell strain and the parental MSU-1.1 TG^R recipient cell strain. As expected, the chromosome donor mouse A9 somatic hybrid cell strains each contained a single fluorescently-labeled copy of human chromosome 11, 12, or 15 and the parental recipient cell strain, MSU-1.1 TG^R,

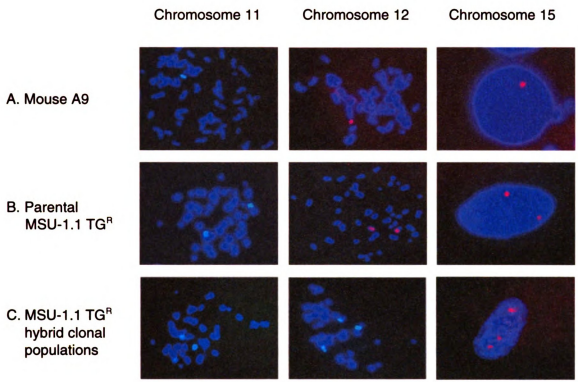

Fig. 1. Fluorescent analysis of the mouse A9 cells carrying human chromosome 11, 12, or 15, parental MSU-1.1 TG^R cells, and the MSU-1.1 TG^R hybrid clonal populations. A. Donor mouse A9 somatic hybrid cell strains. B. Parental MSU-1.1 TG^R recipient cell strain. C. MSU-1.1 TG^R hybrid clonal populations. For chromosomes 11 and 12, whole chromosome painting probes were used on chromosome metaphase spreads. For chromosome 15, a centromeric enumeration probe was used on interphase cell spreads. A representative from each hybrid clonal population is shown.

contained two copies of each of the fluorescently-labeled chromosomes 11, 12, or 15 (Fig. 1). Hybrid clonal populations carrying an additional chromosome 11, 12, or 15 continued to exhibit a normal, spindle-shaped morphology like the parental MSU-1.1 TG^R cells (Fig. 2A).

T24 *H-RAS* transfection and Western blot analysis of MSU-1.1 TG^R hybrid clonal populations. To determine whether the introduction of each chromosome into MSU-1.1 TG^R cells could prevent these hybrid clonal populations from being malignantly transformed, we transfected a minimum of two independent hybrid clonal populations obtained from each chromosome transfer group with the pcDNA/T24 *H-RAS* plasmid and selected for blasticidin resistant cells. Several clonal populations that had a transformed morphology were isolated from each chromosome group. MSU-1.1 and MSU-1.1 TG^R cells, upon overexpression of the *H-RAS* oncogene, are known to exhibit a transformed morphology (3) (McCormick *et al.*, unpublished studies). The non-transformed MSU-1.1 TG^R cells are shown in Figure 2A and the *H-RAS* transformed cells in Figure 2B. The H-Ras protein expression levels were determined by Western blot analysis (Fig. 3). The H-Ras protein expression levels were designated on a scale of "1" through "4"; "1" being equal to the parental MSU-1.1 TG^R cell strain and "4" being equal to the positive control MSU-1.1 TG^R + T24 H-Ras cell strain.

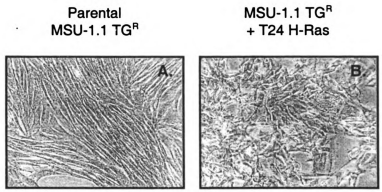

Fig. 2. Morphology of the parental MSU-1.1 TG^R cell strain (spindle-shaped) (A.) and the T24 *H-RAS* transformed MSU-1.1 TG^R cell strain, MSU-1.1 TG^R + T24 H-Ras (transformed) (B.). The spindle-shaped morphology is characterized by long cytoplasmic processes. The transformed morphology is characterized by a rounder, more refractile appearance.

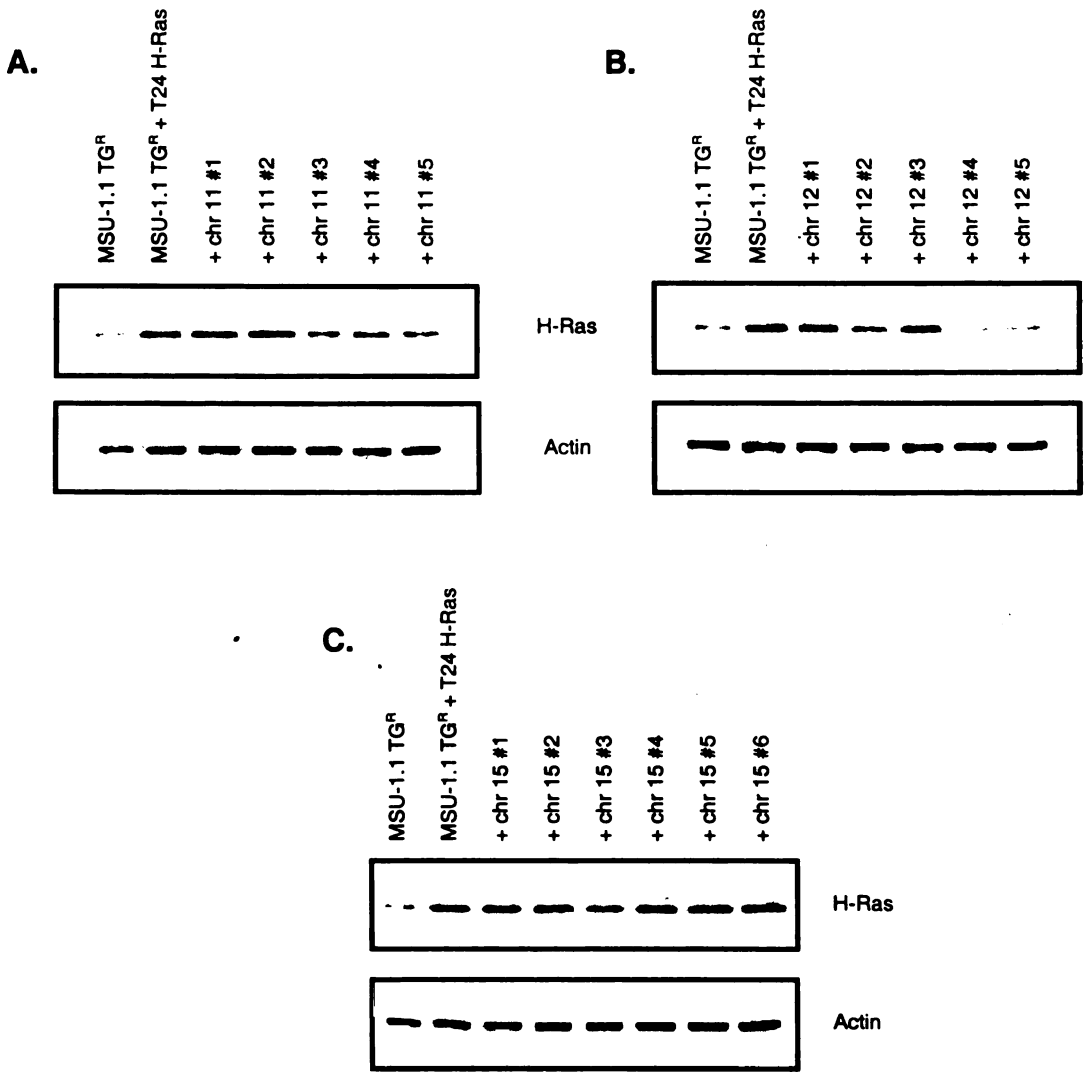

Fig. 3. Western blot analysis for H-Ras (21 kD) protein expression in T24 *H-RAS* transformed MSU-1.1 TG^R chromosome hybrid clonal populations. Actin (42 kD) was used as a loading control. A. Chromosome 11 hybrid clonal populations. B. Chromosome 12 hybrid clonal populations. C. Chromosome 15 hybrid clonal populations. Blots are representative from a minimum of two independent Western analyses using independent protein preparations.

Table 1. Characterization of MSU-1.1 TG^R T24 *H-RAS* transfected chromosome hybrid clonal populations.

	H-Ras Protein Expression Levels [a]	Cell Morphology	# of Tumors Formed/Injection Site [b]	Tumor Latency (weeks) [c]	Maximum Colony Diameter Formed in Agarose (μm)	Cell Doubling Time (h)
Parental MSU-1.1 TG^R	1	Spindle-shaped	0/6	-	<20	36
MSU-1.1 TG^R + T24 H-Ras	4	Transformed	4/4	7	120	19
+ chromosome 11 clonal population						
# 1	4	Transformed	6/6	8	160	16
# 2	4	Transformed	4/6	8	80	21
# 3	2	Transformed	0/6	-	<20	31
# 4	2	Transformed	0/6	-	<20	27
# 5	2	Transformed	0/4	-	<20	31
+ chromosome 12 clonal population						
# 1	4	Transformed	4/4	4	160	17
# 2	3	Transformed	6/6	5	120	23
# 3	4	Transformed	4/4	7	120	22
# 4	1	Transformed	0/4	-	<20	40
# 5	1	Transformed	0/6	-	<20	35
+ chromosome 15 clonal population						
# 1	3	Transformed	0/4	-	140	19
# 2	3	Transformed	0/2 [d]	-	120	26
# 3	2	Transformed	1/4	20	<20	28
# 4	4	Transformed	2/4	8	60	25
# 5	4	Transformed	0/4	-	120	21
# 6	4	Transformed	0/4	-	80	26

[a] H-Ras protein expression levels were designated on a scale of "1" through "4"; "1" being equal to the parental MSU-1.1 TG^R cell strain and "4" being equal to the positive control MSU-1.1 TG^R + T24 H-Ras cell strain.

[b] A minimum of two mice were injected (two sites/mouse) for each cell strain. All tumors that formed were histologically verified as being fibrosarcomas.

[c] Time from cell injection to tumor removal. Tumor volumes ranged from 21 mm^3 to 3703 mm^3, with an average volume of approximately 1000 mm^3.

[d] One animal died during the experimental period for + chromosome 15, clonal population #2.

Tumorigenicity status of MSU-1.1 TG^R hybrid clonal populations. The selected T24 *H-RAS* transfected hybrid clonal populations obtained for each chromosome were tested for their tumorigenicity potential in athymic mice. Hybrid clonal populations for chromosome 11 (#1 and #2) and chromosome 12 (#1, #2, and #3) that expressed the T24 H-Ras protein at high levels ("3" to "4") formed tumors within eight weeks (Table 1). These results were consistent with control studies, in which four of four MSU-1.1 TG^R clonal populations that expressed high levels ("3" to "4") of H-Ras protein formed tumors within eight weeks. In contrast, four of five chromosome 15 T24 *H-RAS* transformed hybrid clonal populations expressing high levels ("3" to "4") of H-Ras protein did not form tumors during the six-month testing period (Table 1). As expected from previous studies involving T24 *H-RAS* transfection into MSU-1.1 and MSU-1.1 TG^R cells (3) (McCormick *et al.*, unpublished studies), chromosome hybrid clonal populations that had a low level ("1" to "2") of H-Ras protein expression did not form tumors or did so with a low frequency and a long latency when injected into athymic mice (Table 1). These results show that introduction of chromosome 15 prevented T24 *H-RAS* malignant transformation indicating that this chromosome has tumor suppressor activity.

Characterization of *in vitro* properties of MSU-1.1 TG^R hybrid clonal populations. To analyze the hybrid clonal populations for *in vitro* properties of transformation as a result of the transfection with the T24 *H-RAS* oncogene, the

hybrid clonal populations were tested for their ability to form colonies in agarose and their growth rate. MSU-1.1 TG^R T24 H-Ras transformed chromosome 11 clonal populations #1 and #2, and chromosome 12 clonal populations #1, #2, and #3 that expressed high levels ("3" to "4") of H-Ras protein were able to form colonies in agarose (greater than 80 μm) and had similar cell doubling times to the positive control cells, MSU-1.1 TG^R + T24 H-Ras (Table 1, Fig. 4, and Fig. 5). The parental non-tumorigenic MSU-1.1 TG^R cells did not form colonies in agarose and grew at a slower rate (Table 1, Fig. 4, and Fig. 5). The chromosome 15, non-tumorigenic, high ("3" to "4") H-Ras expressing hybrid clonal populations also had similar *in vitro* properties (Table 1, Fig. 4, and Fig. 5). All chromosome hybrid clonal populations with low ("1" to "2") H-Ras expression did not form colonies in agarose and grew at similar rates to the parental MSU-1.1 TG^R cell strain (Table 1). These results indicated that the increase in H-Ras protein expression level was sufficient to transform these chromosome hybrid clonal populations *in vitro*, but the introduction of chromosome 15 inhibited the tumorigenicity of the clonal populations that expressed high levels ("3" to "4") of the H-Ras protein. Thus, tumorigenesis was not correlated with the ability to form colonies in agarose or growth rate of these chromosome 15 hybrid clonal populations.

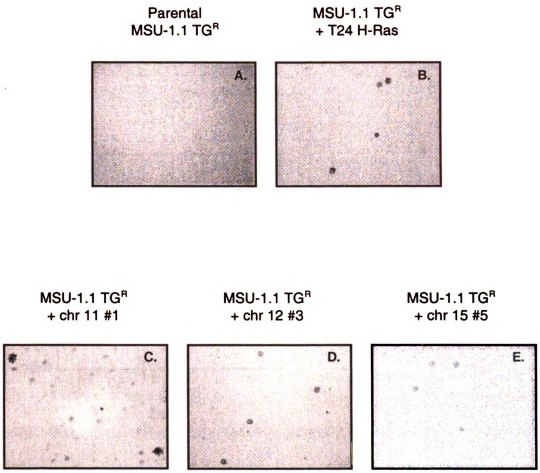

Fig. 4. Cell growth in agarose for representative MSU-1.1 TG^R hybrid clonal populations obtained for chromosome 11, 12, and 15. The non-tumorigenic parental MSU-1.1 TG^R cell strain (A.) does not form colonies. The tumorigenic MSU-1.1 TG^R + T24 H-Ras (B.) and the MSU-1.1 TG^R hybrid clonal populations for chromosome 11 (C.), 12 (D.), and 15 (E.) form colonies.

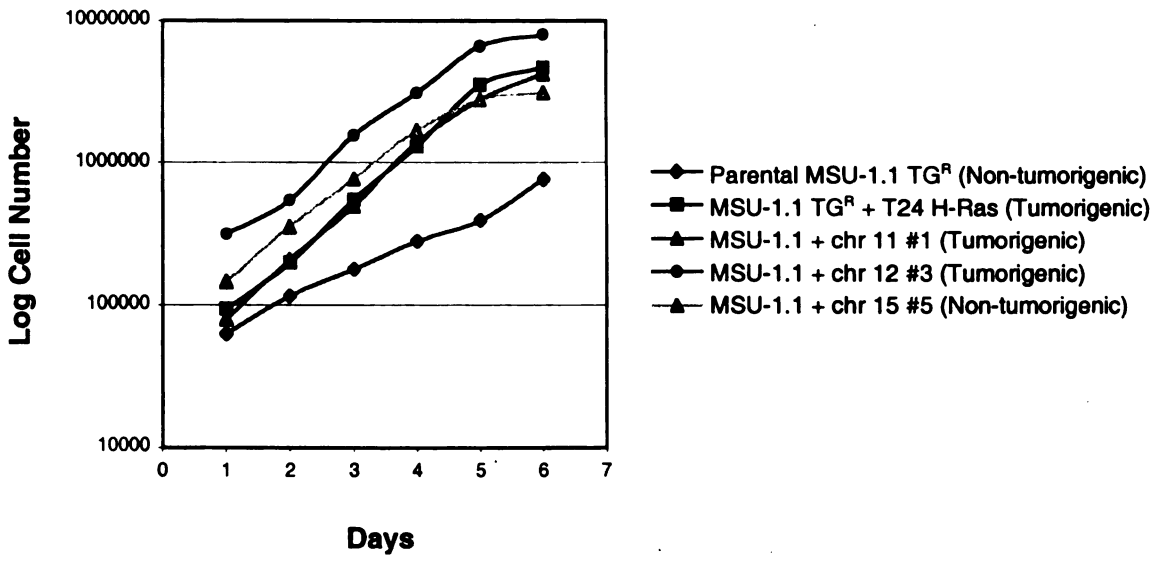

Fig. 5. *In vitro* growth curves for representative MSU-1.1 TG^R hybrid clonal populations obtained for chromosome 11, 12, and 15. The tumorigenicity status of each hybrid clonal population is shown in parenthesis

Chromosome introduction into PH2MT cells and verification of hybrid clonal populations. Based on the above findings, we hypothesized that introduction of chromosome 15 into tumor derived MSU-1.1 cells (PH2MT), previously transformed by overexpression of the H-Ras protein (3), would suppress tumor formation by these cells. To test this hypothesis, we introduced chromosome 11, 12, or 15 into PH2MT cells by MMCT. Several independent hygromycin B drug resistant hybrid clonal populations were isolated for each transferred chromosome from two independent chromosome transfer experiments. Hybrid clonal populations were verified for the presence of the appropriate extra copy of the chromosome by FISH analysis. All hybrid clonal populations examined by FISH analysis were verified as positive for the presence of the appropriate extra chromosome and three verified hybrid clonal populations for each chromosome were used for further study. A representative hybrid clonal population for each chromosome group is shown in Figure 6. FISH analysis was also performed for the parental PH2MT recipient cell strain, and as expected, only two copies of the appropriate fluorescently-labeled chromosome were detected (Fig. 6).

All chromosome 11 and chromosome 12 hybrid clonal populations exhibited a transformed morphology like that of the parental PH2MT cells (Fig. 7A). Surprisingly, the introduction of chromosome 15 into the PH2MT cell strain resulted in two morphologically distinct hybrid clonal populations. Out of the ten hybrid clonal populations obtained for each chromosome 15 transfer, five clonal

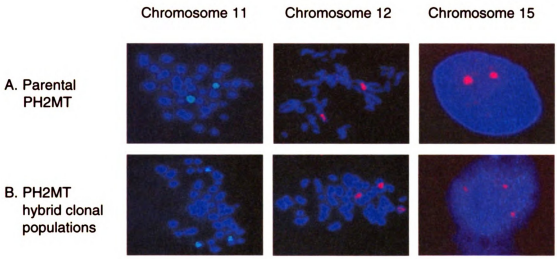

Fig. 6. Fluorescent analysis of the parental PH2MT cells and the PH2MT hybrid clonal populations. A. Parental PH2MT recipient cell strain. B. PH2MT hybrid clonal populations. For chromosomes 11 and 12, whole chromosome painting probes were used on chromosome metaphase spreads. For chromosome 15, a centromeric enumeration probe was used on interphase cell spreads. A representative from each hybrid clonal population is shown.

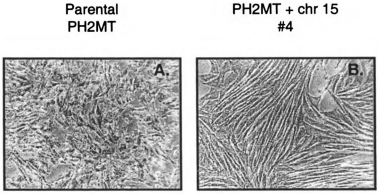

Fig. 7. Morphology of the parental PH2MT cell strain (transformed) (A.) and the PH2MT chromosome 15 hybrid clonal population #4 (spindle-shaped) (B.). The spindle-shaped morphology is characterized by long cytoplasmic processes. The transformed morphology is characterized by a rounder, more refractile appearance.

populations had cells that were morphologically transformed, and five hybrid clonal populations had cells that were spindle-shaped (Fig. 7B). Introduction of chromosome 15 into another (independent) MSU-1.1, T24 *H-RAS* transformed, tumor derived cell strain, also resulted in hybrid clonal populations that consisted of spindle-shaped cells or transformed cells with the same frequency.

Tumorigenicity status of PH2MT hybrid clonal populations. To test if the introduction of chromosome 11, 12, or 15 could suppress tumor formation by the PH2MT cell strain, the hybrid clonal populations were injected into athymic mice. All of the chromosome 11 and chromosome 12 hybrid clonal populations, like the parental PH2MT cells, formed tumors within six weeks of injection (Table 2). Two of three morphologically transformed chromosome 15 hybrid clonal populations (#1 and #2) formed tumors with a short latency and the third clonal population (#3) formed a single tumor with a somewhat longer latency (Table 2). However, two of the three spindle-shaped chromosome 15 hybrid clonal populations (#4 and #5) formed no tumors and the third one (#6) formed only a single tumor after a long latency (Table 2). These results are consistent with the findings obtained from the MSU-1.1 TG^R T24 *H-RAS* transformed chromosome 15 hybrid clonal populations described above in this paper.

Table 2. Characterization of PH2MT chromosome hybrid clonal populations.

	Cell Morphology	# of Tumors Formed/ Injection Site[a]	Tumor Latency (weeks)[b]	Maximum Colony Size Formed in Soft Agarose (μm)	Cell Doubling Time (h)
Parental PH2MT	Transformed	3/4	5	140	17
MSU-1.1	Spindle-shaped	0/6	-	<20	36
+ chromosome 11 clonal population					
#1	Transformed	6/6	6	140	14
#2	Transformed	6/6	6	180	17
#3	Transformed	5/6	4.5	140	13
+ chromosome 12 clonal population					
#1	Transformed	6/6	4.5	140	14
#2	Transformed	5/6	6	100	19
#3	Transformed	6/6	4.5	100	21
+ chromosome 15 clonal population					
#1	Transformed	5/6	5	140	17
#2	Transformed	4/4	6.5	120	16
#3	Transformed	1/4	9	140	22
#4	Spindle-shaped	0/6	-	<20	37
#5	Spindle-shaped	0/6	-	<20	28
#6	Spindle-shaped	1/6	24	60	32

[a] A minimum of two mice were injected (two sites/mouse) for each cell strain. All tumors that formed were histologically verified as being fibrosarcomas.

[b] Time from cell injection to tumor removal. Tumor volumes ranged from 8 mm^3 to 4504 mm^3, with an average volume of approximately 1000 mm^3.

Characterization of *in vitro* properties of PH2MT hybrid clonal populations. To analyze the PH2MT hybrid clonal populations for *in vitro* properties of transformation, the cells were tested for their ability to form colonies in agarose and their growth rate. The morphologically transformed, tumorigenic chromosome 11, 12, and 15 hybrid clonal populations formed colonies in agarose (greater than 100 μm), and had cell doubling times similar to the parental PH2MT cell strain (Table 2, Fig. 8, and Fig. 9). The morphologically transformed, slightly tumorigenic chromosome 15 hybrid clonal population (#3) also had similar *in vitro* transformed properties (Table 2). However, the morphologically spindle-shaped, non-tumorigenic chromosome 15 hybrid clonal populations (#4, #5, and #6) formed colonies in agarose less than 60 μm and had longer doubling times than the parental PH2MT cell strain (Table 2, Fig. 8, and Fig. 9). This observation is consistent with their failure to form tumors (#4 and #5) or markedly reduced tumorigenicity (#6).

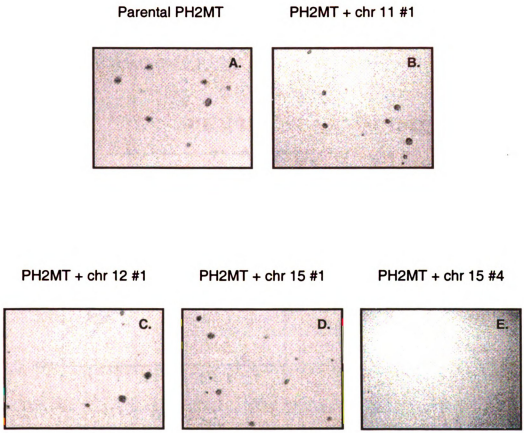

Fig. 8. Cell growth in agarose for representative PH2MT hybrid clonal populations obtained for chromosome 11, 12, and 15. The tumorigenic parental PH2MT cell strain (A.) and the tumorigenic hybrid clonal populations for chromosome 11 (B.), 12 (C.), and 15 (D.) form colonies. The non-tumorigenic, spindle-shaped, PH2MT chromosome 15 hybrid clonal population #4 (E.), does not form colonies.

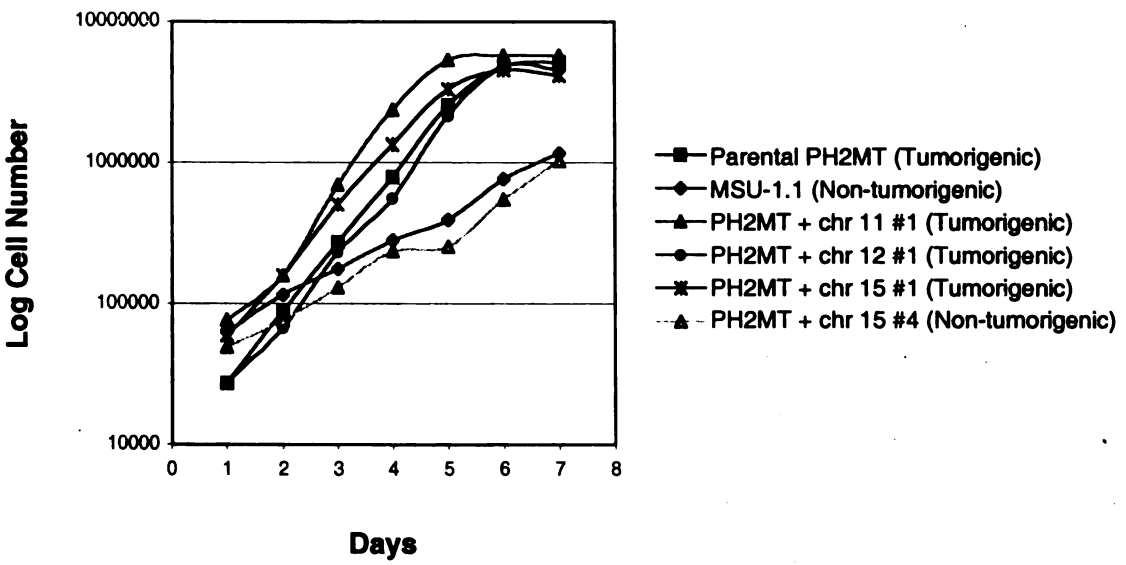

Fig. 9. *In vitro* growth curves for representative PH2MT hybrid clonal populations obtained for chromosome 11, 12, and 15. The tumorigenicity status of each hybrid clonal population is shown in parenthesis.

DISCUSSION

Chromosome 15 introduction inhibits tumorigenicity in human immortal and tumor-derived fibroblasts. We have demonstrated by MMCT studies that the introduction of chromosome 15, but not chromosome 11 or 12, suppressed malignant transformation and tumorigenicity in the cell strains tested. This novel finding was obtained by the demonstration that the T24 *H-RAS* oncogene, when expressed at high levels, did not malignantly transform MSU-1.1 TG^R chromosome 15 hybrid clonal populations. However, MSU-1.1 TG^R chromosome 11 and chromosome 12 hybrid clonal populations with high levels of H-Ras protein expression were malignantly transformed. These results were also supported by the findings that MMCT of chromosome 15, but not chromosome 11 or 12, into the tumorigenic PH2MT cell strain suppressed or reduced the tumorigenicity of these cells in four of the six resultant chromosome hybrid clonal populations characterized. H-Ras protein expression levels, as determined by Western blot analysis, were high for all chromosome 11, 12, and 15 hybrid clonal populations (data not shown).

One possible explanation for this tumor suppressor activity is that a TSG is located on the region of chromosome 15 that is lost (15p11.2→15pter) in the MSU-1.1 cells and cell strains derived from it, MSU-1.1 TG^R and PH2MT. However, no genes are known to be localized to this DNA region. It is also possible that both alleles of a TSG located on the region of chromosome 15 present in these cell strains in two copies (i.e. 15qter→15p11.2) has lost function

due to mutational or epigenetic events. The introduction of chromosome 15 could restore the normal function of this TSG.

Of the 1700 known genes located on this chromosome, two are candidate genes that might account for the results presented here. Thrombospondin (*THBS1*) is located at 15q15 (63) and has been shown to play a role in carcinogenesis. *THBS1* negatively regulates cellular proliferation and adhesion, and it also inhibits tumor metastasis and angiogenesis *in vivo* (64). MMCT of chromosome 15 into the human skin carcinoma cell strain, SCL-1, resulted in increased tumor latency associated with decreased tumor vascularization, which was caused by expression of *THBS1* (28, 65, 66). There was no change in *in vitro* growth rates of the chromosome hybrid clones compared to the parental cell strain. Similar results have been reported in *THSB1*-transfected human cutaneous squamous cell carcinoma cell strains (67) and *c-JUN* malignantly transformed rat 3T3 fibroblasts (68) and *v-SRC* malignantly transformed mouse 3T3 fibroblasts (69). Taken together, these recent published studies (28, 65-69) show that *THBS1* plays an important role in *in vivo* inhibition of tumor cell metastasis and angiogenesis, but had no effect on *in vitro* properties such as cell growth rate and ability to grow in agarose in the cells tested. This is in contrast to the findings presented in this study. Current studies are ongoing in the laboratory to investigate the possible role of *THBS1* in the chromosome 15 hybrid clonal populations.

The second candidate gene, *RBP21* (mapped to 15q21.1-21.3), is a pRb-associated protein that functionally interacts with pRb and is thought to be involved in the cell regulatory effects of pRb (70). pRb is a phosphoprotein involved in cell cycle regulation and apoptosis, and it is mutated in a variety of cancer types (71, 72). Since there is little known about *RBP21* function, this novel gene cannot be ruled out as a putative TSG at this time.

Possible targets of chromosome 15 tumor suppressor function. Consistent with an earlier study from this laboratory, the critical factor in the morphological and malignant transformation of the MSU-1.1 cell strain to the PH2MT cell strain was overexpression of the H-Ras protein (3). Therefore, the introduction of chromosome 15 might be suppressing H-Ras protein expression, post-translational modifications of H-Ras, or a downstream effector of H-Ras. Since the PH2MT cell strain is derived from the MSU-1.1 cell strain and both strains are karyotypically identical (3), it is likely that suppression is occurring via the same mechanism in both MSU-1.1 TG^R and PH2MT chromosome 15 hybrid clonal populations. Western blot analysis for the non-tumorigenic MSU-1.1 TG^R T24 *H-RAS* transfected and PH2MT chromosome 15 hybrid clonal populations demonstrated that expression levels of the H-Ras protein were high, comparable to the tumorigenic MSU-1.1 TG^R T24 *H-RAS* transfected and PH2MT chromosome 11 and 12 hybrid clonal populations, suggesting either post-translational modifications of H-Ras or a downstream effector of H-Ras could be

targeted by a gene product located on chromosome 15. Although H-Ras function could be a target, any gene change in the development of the MSU-1.0 cells, and the MSU-1.1 cells derived from them, that contributes to the transformed properties of the MSU-1.1 T24 *H-RAS* transformed cells could be a target.

Chromosome 15 introduction into MSU-1.1 TG^R cells did not prevent morphological transformation as a result of T24 H-Ras protein overexpression. Because we were initially testing the ability of T24 H-Ras protein overexpression to malignantly transform the hybrid clonal populations, only those clones that had cells with a transformed morphology (an indication of H-Ras protein overexpression) were isolated. Based upon the subsequent findings that chromosome 15 introduction into PH2MT cells resulted in non-tumorigenic, morphologically spindle-shaped cells, we hypothesize that chromosome 15 introduction into MSU-1.1 TG^R cells prevented the morphological transformation in some of these hybrid clonal populations by T24 H-Ras protein overexpression, but these clonal populations were not isolated because we were focused on cells that had a transformed morphology.

Chromosome 15 introduction did not suppress tumorigenicity in all chromosome hybrid clonal populations. These findings are consistent with studies in which introduction of chromosome 11 into the human cervical carcinoma cell strain, HeLa, resulted in tumorigenic and non-tumorigenic hybrid

clones (32). The investigators were able to identify the *HTS1* gene as the gene causing tumor suppression and they showed that *HTS1* expression was correlated with the tumorigenicity status of the various chromosome 11 hybrid clonal populations. In addition, some studies have demonstrated that the MMCT procedure can result in the introduction of a chromosome with discrete intrachromosomal deletions and rearrangements that can influence the properties of the resultant hybrid clonal populations (19, 27, 39).

Conclusion. The findings presented here provide evidence for tumor suppressor activity localized to chromosome 15, suggesting that there is a TSG located on this chromosome. The chromosome 15 tumorigenic and non-tumorigenic hybrid clonal populations obtained in this study provide us with a model system to examine these possibilities by comparing the gene expression of the two types of hybrid clones to identify the gene associated with the tumor suppression effects shown for chromosome 15.

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