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In vitro multistage carcinogenesis: A balance of genetic alterations and selective pressures

Schneider, Brandt Lawrence, Ph.D.

The University of Arizona, 1993

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IN VITRO MULTISTAGE CARCINOGENESIS: A BALANCE OF GENETIC ALTERATIONS AND SELECTIVE PRESSURES

by

Brandt Lawrence Schneider

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A Dissertation Submitted to the Faculty of the DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
In Partial Fulfillment of the Requirements For the Degree of Doctor of Philosophy
In the Graduate College
THE UNIVERSITY OF ARIZONA

1993
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Brandt Lawrence Schneider entitled *In Vitro Multistage Carcinogenesis: A Balance of Genetic Alterations and Selective Pressures* and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy/Microbiology & Immunology.

*Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.*

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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DEDICATION

The content and meaning of this dissertation is dedicated to Dingo. He lived life the way it should be lived, with vigor and purpose. Like a wise man once said, he never looked back, so he couldn't have known what caught him.
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ABSTRACT

The long latency of human tumors and the difficulty of transforming human cell lines has necessitated the investigation of rodent models which have helped propagate a general theory of carcinogenesis. This author has addressed the temporal and physiological significance of oncogene activation and tumor suppressor loss during malignant progression of nontumorigenic murine keratinocytes. The data presented here indicate that mechanisms of carcinogenesis may be dependent upon both the model system studied, and on innate environmental selective pressures.

7,12-dimethylbenz[a]anthracene initiation of murine keratinocytes resulted in tumorigenic phenotypes with transferrable dominant transforming activity. However, mutated Ha-ras alleles were not detected in the tumorigenic cell lines despite the direct correlation between chemical initiation and ras activation in vivo.

Somatic cell hybrid studies implicated the loss of multiple tumor suppressors in the progression of a normal cell through a benign state to frank malignancy. While all hybrids were suppressed to varying degrees, nontumorigenic cells suppressed tumor formation to a greater extent than benign cells. Interestingly, a well differentiated carcinoma cell line also suppressed tumor formation. The implication of tumor suppressor loss in the formation of a benign papilloma was a novel finding. Results from these experiments revealed that disruption of differentiation programs is concomitant with tumorigenicity, and that the ability to differentiate in vitro is a prerequisite for tumor suppression in vivo.
Indicative of an inability to differentiate, benign and malignant cell lines failed to express keratins K1 or K10 in response to high extracellular calcium concentrations. While differential northern analysis revealed that keratins K6 and K18 were predominately expressed by either non-tumorigenic or malignant cells, respectively, subtractive hybridization identified five putative gene sequences (Suz 1-4 and 6) expressed exclusively by non-tumorigenic cells.

In summary, somatic cell hybrid studies helped assess the phenotypic dominance of a given stage of cancer. The isolation of genes whose expression is abrogated in malignant cells may further our insight into the role of gene loss during malignant conversion. Finally, a better understanding of the mechanisms of endogenous and exogenous selective pressures will enable the design of improved in vitro models of carcinogenesis.
V. AN OVERVIEW OF MULTISTAGE SKIN CARCINOGENESIS

"Why if man can by patience select variations most useful to himself, should nature fail in selecting variations useful, under changing conditions of life, to her living products?"

Charles Darwin
The Origin of Species, 1859

"The method of science is the method of bold conjecture and ingenious and severe attempts to refute them."

Karl R. Popper
Objective Knowledge: An Evolutionary Approach

Introduction

The trail of cancer is woven throughout history. Today the need for a better understanding is not simply a scientific curiosity. Cancer will claim more victims this year than ever before, and the incidence rate is steadily increasing. Progress and knowledge have come from discoveries of both the apparently minuscule and the obviously omnipotent. Despite the rush of innovations and revelations in the last two decades, neither the disease nor study of carcinogenesis is contemporary. Cell biologists have studied the intricacies of cancer for over 100 years. Today molecular biologists
wrestle with both the cause and the origin of cancer. However, a great many advances today are due to reflection on old problems, dormant hypotheses, and forgotten evidence. Indeed the theories and results from a collection of different scientific disciplines have helped lay the very foundation of molecular biology. The concepts proposed by Darwin, Mendel, Watson & Crick, and others while astounding in their own right, have provided the impulse not for further diversification, but rather unification. While scientists assemble under disciplines labeled cell biology, biochemistry or molecular biology to study carcinogenesis, a new assembly is being sent forth to conquer not by division, but rather by assimilation. More and more it is becoming apparent that to achieve a greater understanding, a gestalten philosophy must be adapted towards not only the whole organism, but also towards the cell, the molecule, and the very environment in which everything finds itself. The principles of pioneers like Darwin or Mendel are becoming recurrent themes in biology. In the study of cancer, like the study the of many natural phenomena, the truth we seek lies not in the aberrancy of the disease, but in subtle secret workings of the normal cell.

Cancer was well known to the Egyptians (Ruddon, 1988). Their writings and mummies have preserved for time some its ravaged victims. The word itself is derived from the greek word karkinos for the crab. It is supposed the disease was so named because its insidious invasive growth pattern often resembled the multi-appendage crab. Early civilizations believed that tumors were plagues sent upon them by God. Not until Hippocrates, the father of medicine, was cancer first ascribed to a natural origin (Tannock
and Hill, 1987). He proposed that an imbalance of bodily humours resulted in malignancy. Fortunately he was incorrect, but from his observation on, people have sought a worldly cause for the disease. Writings from the Middle Ages describe cancer families and cancer villages (Tannock and Hill, 1987). It was becoming apparent that not only was the origin of cancer secular, but that its pattern of incidence was non-random.

Pathologically, cancer is a general name given to a broad group of diverse neoplastic diseases. It is typified by a new abnormal growth. Typically cancers display uncontrolled patterns of proliferation and aberrant differentiation programs. Cancer cells commonly have structural and physiological abnormalities, and cancer cells are frequently aneuploid resulting in a larger nucleus and increased nuclear to cytoplasmic ratios. Tumors often have mitotic indices higher than surrounding normal tissue, and their cellular morphology is substantially more variable. Neoplastic cells may partially resemble normally differentiated tissues, however, they function abnormally both quantitatively and qualitatively. Tumors may be either benign or malignant, but ultimately most neoplasms expand, invade, and destroy surrounding tissues. Eventually, many tumors malignantly metastasize, and treated or not half of all cancers kill their hosts (Alberts et al., 1987).

From a cell biologist's point of view, cancer is an aberration in a somatic cell’s normal program of division and differentiation. In its unperturbed state, every eucaryotic somatic cell lives within a tightly regulated program. Each cell responds to temporal, spatial, and developmental cues with a myriad of actions from the most primeval to the
highly intricate and elaborate. These actions include proliferation, differentiation, and every specialized function imaginable, all orchestrated together in a continuing drive to propagate the species. Natural selective pressures direct this action/response adaptation and genetic imprinting propels the process of evolution. Despite the drive to survive and propagate, every normal cell possesses one inescapable command. From its first division to its last, every cell is committed to die. In a great simplification, an organism can be thought of as a large population of somatic cells dedicated to the survival of a relatively few germ cells. An organism much like a flask of bacteria is a clonal population, but unlike the free-living bacteria, this population of cells behaves in a peculiar manner. The cells of the organism collaborate to survive: altruism for the good of the whole overrides competition. In its unperturbed state, each cellular program ensures that proliferation and differentiation occur in a manner consistent with the survival of the organism. Natural selection, however, applies to all populations: finches on the Galapagos islands, bacteria in a flask, or somatic cells in an organism. Like gravity, natural selection is an ever present invisible force. Any alteration in a cell’s potential confers an advantage or disadvantage that can endanger the entire organism’s collaboration for survival. Selective pressures enrich the competitive spirit of nature. Cells disadvantageously affected die while those advantageously affected proliferate and propagate selfish competition. Populations expand with no apparent usefulness to the host whereas necessary populations are out competed and lost. The delicate balance between cooperation and survival is broken. This is cancer.
**Epidemiology of Human Cancer**

While always a feared and dreaded disease, the significance of cancer has never been so poignant as it is today. Six million people in the world developed cancer in 1987 (Alberts et al., 1987). The number of people dying from cancer has quadrupled since 1900 (Prescott, 1986). In this year alone 1,170,000 new cases of cancer will be diagnosed (Boring et al., 1993). Nearly half of these people will die of their cancer. The statistics are astounding. Cancer affects all of us. One out of every four people we know will develop at least one form of cancer, and in our lifetimes, 25% of all Americans will die of cancer (Prescott, 1986). Many cases of cancer go undiagnosed and unreported, nonetheless it is the second leading cause of death in our country (Boring et al., 1993). Tragically cancer is also the second leading cause of death of our children under the age of 15 (Boring et al., 1993). It is the leading cause of death in women from 35-74 years of age (Boring et al., 1993). Nearly 600,000 Americans died of cancer in 1989 as contrasted to 0.9% of our population that died of complications from AIDS (Boring et al., 1993). The prognosis is less than excellent.

Dramatic strides, however, have been made in some areas. Cancer related deaths from stomach cancer are dramatically lower in both men and women. Nearly three times fewer women die of breast cancer today. Successful treatment of cancer was been especially effective in children under the age of 15. Sixty-eight percent of these children have survival rates exceeding five years (Boring et al., 1993). Only 28% of children
under 15 survived 5 years in 1960 (Boring et al., 1993). This effect is most dramatic in leukemias where survival rates are 10 to 18 as great as 1960 figures (Boring et al., 1993). Still cancer is a great killer of our older populations. More than half of our projected 1993 cancer deaths will be people over 55 years of age (Boring et al., 1993). Most strikingly, the incidence of prostate cancer is up 80% from just 1986 (Boring et al., 1993). Breast cancer incidence is up 48% in the same time period (Boring et al., 1993). Cancer poses a very real threat now to us and to our children.

Evidence and arguments from epidemiology suggest a brighter horizon is possible. In fact, some epidemiologists claim that individual lifestyles account for about 80% of all malignancies (Ruddon, 1988). Smoking, diet, alcohol, and sexual patterns have been shown to be correlated with tumor formation. In some cases, this correlation is great. Thirty-five percent of all tumors are thought to be related to diet and 30% to smoking (Ruddon, 1988). In the most optimistic sense, this suggests that at least one-half of all malignancies are avoidable. Epidemiologists point to lowered cancer incidence, where causative carcinogens have been identified, as proof. It is becoming increasingly evident that cancer incidence segregates with immediate cultural and geographical lifestyles and not intrinsic nationality. At the very least these data are beginning to identify etiological agents of cancer causation.

Neoplastic cells can arise from any mitotically active tissue of the body. Human tumors can be either benign or malignant and are classified by four characteristics: 1) anatomical site, 2) tissue and histological classification, 3) grade of malignancy, and 4)
extent of metastasis. Most significantly, 78% of the new cancers in 1993 and 73% of cancers deaths will be from cancers of the epithelia (Boring et al., 1993). These malignancies include all major sites of tumor formation: digestive tract, respiratory tract, breast, and genital organs. All other non-epithelial cancers make up only 22% of the total cancer incidence (Boring et al., 1993). The focus of this thesis is on a murine epidermal model of multistage carcinogenesis. Chemical initiation and transformation of primary keratinocytes in culture generated benign and malignant phenotypes very similar to human squamous cell tumors of the head and neck. This model and the data presented here illustrate some of the molecular and genetic changes associated with carcinogenesis, and support a mechanistic role for tumor suppressor genes in both the initiation and progression of malignancy. Model systems like this one may someday aid in the development of successful cancer intervention therapy.

**Historical Perspectives**

Molecular biology should not be viewed as an independent entity or even a separate discipline. It is the natural consequence of hundreds of years of biological research. Molecular biology is both a result of and amalgamation of cell biology, biochemistry, and genetics. Today like hundreds of years ago, the driving force behind experiments are the same basic questions of how, when, and why cells proliferate, divide, and die. While each of the four disciplines above originated independently, it is becoming increasingly
apparent that the day to day tools and knowledge of each is indispensable to every other. The foundation of multistage carcinogenesis lies in the origin of each discipline mentioned above, and its future lies in the assimilation and melding of each field into a common and greater understanding.

Cell biology literally got its start over 300 years ago when Robert Hooke described the first cell in a section of cork (Curtis, 1984). The cell theory itself came to life in the middle of the 19th century. In 1838, Matthias Schleiden proposed that all plant tissue was composed of individual cells (Curtis, 1984). In the next year Theodor Schwann recognized that all animal tissues were also composed of cells. He concluded that cells were the essence and basis of life. Twenty years later Rudolf Virchow proposed "Where a cell exists, there must have been a preexisting cell, just as the animal arises only from an animal and the plant only from a plant . . . throughout the whole series of living forms, whether entire animal of plant organisms or their component parts, there rules an eternal law of continuous development." (Curtis, 1984). The effect of his conclusion of biology and eventually on carcinogenesis would be profound.

Biochemistry like chemistry is intimately related to physics. The turn of the 20th century brought new insight into the inner workings of the atom. This knowledge provided a greater understanding of the formation of simple molecules. Linus Pauling and Lawrence Bragg helped elucidate basic molecular structures (Gribbin, 1987). Sergei Ochoa and Arthur Kornberg unveiled multipartite respiratory pathways of the cell, and discovered the phenomenon of biological polymerization (Kornberg, 1975). Cell
biologists, biochemists, geneticists, and physicists were beginning to understand the very nature of heredity. The significance of DNA and the coming of a new scientific age was at hand.

The discoveries of Walther Flemming and Friedrich Miescher foreshadowed the coming of recombinant DNA technology. In 1879, Flemming noted that certain thread-like cellular structures had a high affinity for colored dyes (Gribbin, 1987). He named them chromosomes. He also observed that during cellular division, a process he named mitosis, chromosomes associated with one another, and that each daughter cell generally received an equal number. The notion that chromosomes might possess the genetic material was born. Miescher helped establish that chromosomes were made up nucleic acid (Gribbin, 1987). Separately Griffith, and Oswald and Avery demonstrated that nucleic acid, deoxyribonucleic acid or DNA to be exact, was indeed the cellular hereditary substance (Curtis, 1984). In 1953, Watson and Crick solved the structure of DNA and proposed a mechanism for its replication (Watson and Crick, 1953). The basis for heredity had been established.

Forty years earlier, well before a genetic medium for heredity had been established, Theodor Boveri postulated on the origin of cancer (Boveri, 1914). Boveri's theory proposed that cancer arose from single cells as a result of "wrongly" combined chromosomes. The concept of somatic cell mutation was conceived. Boveri even suggested that these "wrongly" combined chromosomes resulted in a rapidly proliferating cell capable of transferring this trait to its descendants. With the discovery of the
structure of DNA, the concept of wrongly combined chromosomes took on a physical definition. Mutations could be defined chemically.

Chemical Carcinogenesis

Like Boveri, many significant observations regarding the cause of carcinogenesis were made prior to discovery of DNA. By the 18th century, certain chemicals were strongly associated with the induction of malignancy (Tannock and Hill, 1987; Miller, 1978). John Hill connected nasal cancers with snuff use, and Sir Percival Pott reported a high incidence of scrotal cancer in chimney sweeps associated with exposure to soot and tar (Tannock and Hill, 1987; Remond, 1970; Miller, 1978). By the 19th century, coal tar and paraffin oils were strongly associated with skin cancers (Miller, 1978). We now know that these products contain polycyclic hydrocarbons, highly mutagenic and carcinogenic compounds (Miller, 1978; Boutwell and Riegel, 1990). "Aniline" dyes became strongly associated with bladder cancers late in the 19th century (Miller, 1978). These observations led to the first attempt to recreate carcinogenesis in experimental animals. Fischer induced proliferative benign lesions on the skin of rabbits with application of the azo dye scarlet red (Fischer, 1906; Miller, 1978). The lesions did not progress to malignancy and regressed when treatment was discontinued. Yamagiwa and Ichikawa followed this experiment by successfully inducing malignant carcinomas on the ears of rabbits (Haddow and Kon, 1947; Miller 1978). By the early 1920's, Tsutsui and
Passey had shown that extracts of coal tars were carcinogenic to mouse skin (Haddow and Kon, 1947; Miller 1978). The era of recreating human carcinogenesis in the experimental mouse had commenced.

The discovery that many different compounds were carcinogenic to both rodents and man led to an investigation for the active chemical agents. Chemical studies by Bloch, Dreifuss, Kennaway and others led to the identification of the first known class of synthetic carcinogens—polycyclic aromatic hydrocarbons (Haddow and Kon, 1947; Kennaway, 1925; Miller, 1978). Interestingly, the first compound identified was dibenz[a,h]anthracene a close relative to the synthetic carcinogen used in the work presented, 7,12-dimethylbenz[a]anthracene (Kennaway and Hieger, 1930; Miller, 1978).

Early experiments in mouse skin suggested that repetitive treatment with synthetic carcinogens could result in either benign or malignant tumors. The first indication that tumor formation might be a multistage process was published in 1924 by Deelman (Deelman, 1924). He reported that wounding animals pretreated with carcinogenic coal tars resulted in increased tumor incidence. In 1939, Twort and Twort substantiated this by demonstrating that oleic acid, which induces epidermal hyperplasia, enhances tumor incidence (Twort and Twort, 1939). By 1944 Peyton Rous had coined the terms "initiation" and "promotion" to describe discrete stages of carcinogenesis (Friedwald and Rous, 1944). Berenblum first demonstrated promotion with croton oils (Berenblum and Shubik, 1941). Mottram experimentally substantiated the concept of initiation in 1944 (Mottram, 1944). Subsequently Boutwell, Hecker, and others operationally and
experimentally defined genetic and epigenetic events associated with multistage carcinogenesis (Boutwell, 1964; Hecker, 1978; Slaga.).

**Mechanistic Theories of Chemical Carcinogenesis**

Boveri stated the first albeit general mechanistic theory of carcinogenesis. In 1928, Bauer proposed the mutational theory (Bauer, 1928). This theory suggested that heritable subtle changes in a cell’s genome were responsible for a cancer cell’s altered attributes. Well ahead of its time, experimental proof of this theory was unattainable at the time. Most mechanistic theories of chemical carcinogenesis at this time proposed a physical interaction between cellular entities and chemical moieties. In another perhaps exceptional intuitive leap, the Millers proposed the deletion hypothesis (Miller and Miller, 1947; Pitot, 1990). They stated that deletion of a critical protein or proteins were responsible for the development of neoplastic cells. While the result of this hypothesis can be appreciated in light of today’s tumor suppressor gene hypotheses, at the time the Millers proposed an interaction directly between the chemical carcinogen and proteins. Nearly twenty years later DNA became the focus. The Millers demonstrated that electrophilic intermediates were the ultimate carcinogenic forms of chemical carcinogens (Miller and Miller, 1966; Pitot, 1990). Subsequently free radical intermediates were also demonstrated to be highly carcinogenic. Bruce Ames first showed that carcinogens were mutagenic (Ames et al., 1973; Ames and McCann, 1976). A direct interaction between
chemical carcinogens and DNA was thought to cause cancer. While exactly how this occurs is still a subject of debate, the proposal of the oncogene hypothesis centered proposed mutagenic activity onto a given class of genes. Previously, aberrations in DNA repair activities or DNA polymerase fidelities had been proposed as the cause of malignancy (Pitot, 1990). However, neither mechanism seemed specific enough to account for the changes observed. In 1969, Huebner and Todaro stated that retroviruses could contain cancer causing genes (Huebner and Todaro, 1969). The term oncogene was born. The hypothesis gained momentum by discoveries that viral oncogenes were really only mutated cellular genes (Der et al., 1982; Santos et al., 1982; Parada et al., 1982). The discovery that both human tumors and chemically initiated rodent tumors contained similarly mutated cellular oncogenes has led to the proposal that oncogene activation was sufficient for tumor initiation (Bishop, 1987; Bishop, 1991; Balmain and Pragnell, 1983; Balmain et al., 1984; Brown et al., 1986; and others). Nearly eighty years later molecular biologists believed they had found the subtle genomic changes Bauer had described and Boveri had labeled wrongly combined chromosomes. Oncogene activation was regarded as the cause of malignancy (Brown et al., 1986; Bishop, 1987; Kumar et al., 1990; Weinberg, 1985; Balmain and Brown, 1988).

Initiation of Carcinogenesis and Oncogene Activation

Since the early 1940's, skin carcinogenesis has been described as a multifaceted
process. Early investigations by Rous and co-workers demonstrated that local irritation enhanced the process of tumor formation. In 1944, Friedwald and Rous proposed the two stage initiation / promotion theory of skin carcinogenesis (Friedwald and Rous, 1944). Initiation was described as the event which initialized a normal cell to form latent tumor cells. By the 1960's the two-stage model was acknowledged to contain a third stage termed progression (Foulds, 1954; Pitot, 1990). The model became known as the multistage theory of carcinogenesis.

Today multistage carcinogenesis is well established in both rodent and human models (Balmain and Brown, 1988; Barrett and Fletcher, 1986; Barrett and Anderson, 1993; Bishop, 1991; Vogelstein and Kinzler, 1993). It is often operationally divided into three distinct stages—initiation, promotion, and progression. This division is particularly useful for illustration and discussion purposes, but experimentally, each stage is unlikely to be discrete or singular. At least several phases or functional changes are likely to be associated with each stage. Furthermore, while a pathological state is frequently attributed to each defined stage, in actuality a broad range of pathological phenotypes replace this clear distinction. In fact, outside of experimental model systems, the sequence, order, and necessity of particular stages may be dispensable.

The process of initiation of mouse skin is proposed to be an irreversible event that predisposes cells to tumorigenicity (Slaga, 1983). While the subject of this work and many other experimental models pertains to chemical carcinogenicity, it should be stressed that physical, viral, or radiation insults can also initiate malignancy (Tannock and
Hill, 1987). The irreversible nature of the initiation event insinuated the involvement of a heritable genetic change. The oncogene hypothesis and subsequent identification of cancer-causing genes set the stage for a genetic definition of the initiation of malignancy. Mariano Barbacid first described a genetic alteration attributed to a chemical carcinogen (Zarbl, 1985). Rat mammary tumors derived from chemical initiation contained a mutated cellular oncogene (Zarbl, 1985). This gene was the cellular homolog of the viral Rous avian sarcoma gene. The implication was clear: viruses and chemical carcinogens initiated the tumorigenic process by altering cellular genes. Subsequent gene transfer experiments determined that at least two oncogenes were required to transform primary rodent cells in culture (Land, 1983). Virally transferred mutated ras oncogenes followed by classical tumor promoters results in benign tumors in mice (Brown, 1986). Tumor initiation became synonymous with oncogene activation (Brown et al., 1986; Bishop, 1987; Kumar et al., 1990; Weinberg, 1985; Balmain and Brown, 1988). Significant questions began to be asked about the nature of tumor promotion. What defined tumor promotion? What were the molecular mechanisms? What genes were involved?

**Tumor Promotion and Selective Pressures in Carcinogenesis**

The concept of tumor promotion evolved from studies using subcarcinogenic doses of chemical initiators (Mottram, 1944; Berenblum and Shubik, 1941). The concentrations of chemical initiators sufficient to induce tumor formation alone were determined
experimentally. In such cases, these chemical initiators were said to be acting as complete carcinogens. Doses below this critical concentration, however, did not result in tumor formation unless other agents were involved. Subsequent to initiation, physical insults, wounding, or further chemical treatment elicited tumor formation and were said to be acting as tumor promoters (Berenblum and Shubik, 1947; Boutwell, 1964; Stenback, et al., 1974). The discovery that chemical initiation was a rapid event with no distinguishing pathological changes seemed to be in contrast to the relatively long latency periods of most tumors. In humans, tumor latencies can be as long as four decades. This implies that the promotion stage of tumor formation is the rate limiting step and not tumor initiation.

Early studies with tumor promotion indicated that promoters acted to enhance the effect of the original tumor initiator (Berenblum, 1941). In fact, these results made it tempting to speculate that the correlation between tumor potentiation and tumor promoters was due to a direct interaction between the initiating compound and the tumor promoter. Importantly, any direct interaction like this would serve to potentiate tumor formation through the role of acting as a co-carcinogen. 1,1,1-trichloroprop-2-ene behaves in such a manner by repressing epoxide hydrase and intracellular glutathione. Indeed, the majority of tumor promoters are co-carcinogens (Berenblum, 1941; Miller, 1978). In these experimental model systems, however, treatment of uninitiated skin rarely or never results in tumor formation. Important exceptions to this will be discussed in another chapter (Pelling, 1988). Typically mouse skin tumor promoters do not bind DNA and are
not mutagenic, and apparently there is no correlation between tumor promotion activity and inhibition of DNA repair. On the surface then, it appears as though tumor promoters work entirely independent of tumor initiators.

Initiation requires only a single exposure to a carcinogen (Slaga; Colburn and Boutwell, 1966). In the mouse skin system, not only is this effect irreversible but it displays a temporal permanency (Berenblum and Shubik, 1949; Scribner and Suss, 1978). In fact, the tumor initiation event appears to be registered in the epidermis of the animal for its entire lifetime (Van Duuren et al., 1975; Van Duuren et al., 1978). Van Duuren et al. have shown that 56 weeks after chemical initiation, mouse skin still remains sensitive to tumor promotion despite the fact that histologically we know that the epidermis is constantly self-renewing (Van Duuren et al., 1975; Van Duuren et al., 1978).

In contrast, not only is tumor promotion reversible, but it is only dose dependent if the doses are given in regular intervals. Boutwell et al have shown that increasing the time between successive promoter doses beyond a certain critical time eliminates the tumor promoting activity even if the same cumulative dose is given (Boutwell, 1964). All of these results point to two significant questions: 1) What is the initiated cell population?, and 2) what is the mechanism of tumor promotion?

Historically, croton oil is the first known tumor promoter and remains today one of the most studied mouse skin tumor promoters. Hecker and Van Duuren simultaneously reported the isolation of the active compound in croton oil (Hecker, 1962; Van Duuren et al., 1963; Van Duuren et al., 1965). These compounds are derived from a diterpene.
alcohol of phorbol esters; the most potent of which is phorbol myristate also known as
12-O-tetradecanoylphorbol-13-acetate or TPA (Van Duuren, 1969; Hecker, 1968; Hecker,
1971; Van Duuren and Sivak, 1968). For the remainder of this section, TPA and the
mouse skin system will be discussed as a typical model of in vivo tumor promotion. The
action and mechanism of tumor promotion will be discussed in the context of the two
questions raised earlier.

TPA is a hyperplastic agent, and early research indicated that it interacted with the
cell membrane (Van Duuren, 1969; Fisher et al., 1979). Today we know much more
about the molecular mechanism and signal transduction pathways of TPA. The specifics
of TPA's interaction with its receptor and the immediate down stream responses have
been greatly clarified, however, a specific understanding of its activity escapes us. It is
known that TPA interacts with and activates protein kinase C (Weinstein, 1981; Castagna
et al., 1982; Ashendel et al., 1983; Kraft and Anderson, 1983; Niedel et al., 1983).
Subsequent work has shown that this signal transduction pathway activates the nuclear
oncoproteins fos and jun which in turn transactivate other known and unknown genes
(Lewin, 1991). Several families of genes are regulated by fos and jun AP-1 complex, of
which the metalloprotease family is the most studied (Matrisian. 1991; Liotta et al.,
1991). While TPA definitely induces the transcription of the metalloprotease family, it
is less clear what role these genes have in tumor promotion. While TPA is a known
hyperplastic agent, it has also been shown to induce epidermal cells to differentiate
(Berenblum, 1954; Raick, 1974; Colburn et al., 1975; Yuspa et al., 1976). The
mechanisms of these actions are less clear. While some of the mechanistic actions of tumor promotion are more understood, a specific mechanism of action has yet to be established. The implication of an irreversible initiation event implies the existence of an essentially immortal cell population in the epidermis. A "stem cell" hypothesis is not unique, however, the nature and proof of these cells remains indefinite. Wholly in vitro carcinogenesis is often used as a model system to simulate in vivo human or rodent carcinogenesis. While the relevancy of such in vitro systems is the subject of a chapter to follow, for the moment I will consider only one relevant comparison. In vitro carcinogenesis is also frequently described as having discrete stages (Weinberg, 1985; Weinberg, 1989; Hunter, 1991). Furthermore, individual oncogenes have been identified as being directly involved in each step: ras activation has been correlated with morphological transformation, and myc activation has been correlated with immortalization (Weinberg, 1985; Weinberg, 1989; Hunter, 1991). Thus in vitro results suggest that the acquisition of immortalization is prerequisite to tumorigenicity. In contrast, in vivo results suggest that ras activation is either an immortalization event or the initiation event only occurs in endogenous immortal cells. It is unlikely that every initiation event confers immortalization. therefore, the second hypothesis is more likely to be true. This apparent paradox will be discussed in more detail in a proceeding chapter, but in the context of this section, the role of natural selection and micro-evolution will be discussed as the possible general mechanism of tumor promotion. This author and others have proposed that endogenous and exogenous tumor promoters determine the
genotype and phenotype of resultant tumors (Schneider and Bowden, 1992; Alberts, 1989; Harris, 1991; Loeb, 1991; Farber and Rubin, 1991). One of the goals of the work presented here was to present evidence for and to discuss a role for selective pressures in carcinogenesis. Briefly, it is proposed that the adaptability of the initiated cell and permissibility of the environment select the genotype of the resulting tumor cell, and the initial activation frequency of a given proto-oncogene is less important than the subsequent recruitment and contribution of that activated oncogene to a phenotype that is selectively advantageous. This, I propose, is essentially the mechanism of tumor promotion.

**Progression: Cancer as a Disease of Abnormal Differentiation.**

Cancer is a disease of abnormal differentiation. If a tumor's growth remains unchecked it will ultimately directly or indirectly kill its host. In general, the rate of a tumor's growth is determined by the steady state equilibrium between cell death and unregulated division. Most but not all tumors have a higher mitotic index than their surrounding tissues (Ruddon, 1987). Malignant cells that divide at similar rate as compared to surrounding tissues still grow in mass because unlike their normal counterparts, most tumor cells as a population do not differentiate and die (Ruddon, 1987). Nearly every tissue in the body maintains a regenerative capability. This capability appears to serve two functions: 1) it allows for the specialized repair and
restoration of function in the event of physical injury (i.e., a cut heals), and 2) its allows for the generalized process of self renewal. The importance of the first function is self-explanatory, however, the role of wounding in tumor promotion will be discussed in a later chapter. The significance of the second function, self-renewal, is less clear. By analyzing which tissues commonly undergo self-renewal, perhaps the purpose of this function will come to light. Two cell types make up the majority of the self-renewing populations in the body: the hemopoietic system, and epithelial cells. For the purposes of this discussion, only epithelial cells will be considered. Two illustrative examples of epithelial self renewal are the intestinal mucosa and the skin. In either case, the cells of these tissues divide regularly. This growth is balanced by the concomitant death and loss of cells at the outermost surface. This process of self-renewal most likely serves a protective function. Several of the preceding sections have established that chemical and physical carcinogens are intricately involved in the genesis of cancer. By extrapolation, the cells most at risk for cancer likely would be those that have the most frequent contact with chemical or physical insults. A case in point are epithelial cells. For example, the epithelial cells of the skin receive substantially higher doses of UV irradiation than stromal fibroblasts only millimeters below them (Elder, 1990, Ananthwamy and Pierceall, 1990). Logically then, the body could lower its total accumulated insults in the skin if the epithelial cells turned over frequently. As stated above, this is exactly the case. In epithelial cells unlike their tumor counterparts, division is tightly regulated. The primary source of this regulation is through the induction of differentiation.
Differentiation is the term given to the sum of all processes that result in the structural or functional cellular specialization. Self-renewing cells arise from a basal or a "stem" cell pool and ultimately they differentiate into the specialized cells of the tissue. Differentiated cells perform the day to day functions required for the organism to survive. In general, these cells have reached a proliferative dead-end. Under ordinary circumstances, these cells will never divide. They simply function, age, die, and are replaced by identically differentiated cells.

The differentiation state of epithelial cells and its role in carcinogenesis is a major subject of this work. While unregulated proliferation is perhaps the trait most frequently associated with malignancy, increasing evidence points to a central role for the regulation of differentiation in carcinogenesis.

The multistage model of mouse skin carcinogenesis predicts the progression of an initiated cell through a number of discrete stages: initiation, promotion, and progression. Both in vivo and in vitro models have associated alterations in proliferative potential and aberrations in differentiation programs during epidermal carcinogenesis. However, due to the long latency of most cancers of the epithelia, hyperproliferation alone has been postulated to be insufficient to account for tumor formation (Yuspa, 1987). Therefore, defects in the normal differentiation pathway have been proposed as the rate limiting step in epidermal carcinogenesis (Yuspa, 1987). The work presented here concerns primarily the process of keratinocyte differentiation.

The most highly studied molecular marker of keratinocyte differentiation is the
keratin gene family which codes for more than 30 independent proteins (Fuchs, 1990; Watts, 1991; Coulombe, 1993; Steinert, 1993). On the basis of amino acid composition, keratins are grouped into either type I acidic keratins (pKi 4.5-6.0, K9-K19) or type II basic keratins (pKi 6.0-7.5, K1-K8) (Fuchs, 1990; Watts, 1991; Coulombe, 1993; Steinert, 1993). A type I keratin is always expressed with its type II partner, and 10,000-20,000 protein heterodimers are thought to oligomerize to form 10 nm intermediate filaments.

In normal epidermis, the basal cells not only are involved in self renewal but also in the generation of progenitors committed to terminal differentiation. Furthermore, basal cells or cells of the hair follicle are thought to be the targets of chemical carcinogenesis in vivo. Basal cells express predominately K5 and its partner K14 (Fuchs, 1990; Watts, 1991; Coulombe, 1993; Steinert, 1993). The mitotically active outer sheath root cell of the hair follicle, also expresses K5 and K14, but unlike basal cells, these cells frequently express K6, K16, and K19 (Coulombe et al., 1989). Expression of the K6/K16 keratin pair, the hyperproliferative keratins, is often associated with proliferative potential, wound healing, or malignancy (Finch et al., 1991; Kopan et al., 1989). As keratinocytes stratify, migration vertically off the basement membrane correlates with a loss of mitotic activity and commitment to terminal differentiation (Fuchs, 1990). These suprabasal cells cease K5, K14 expression and initiate expression of the K1/K10 keratin pair (Fuchs, 1990). As these spinous cells become further stratified, keratin filaments accumulate and aggregate; loricrin and filaggrin expression contribute to the formation of the cornified envelope. Keratin filaments make up 85% of the protein content of the completely differentiated cell
of the stratum corneum (Fuchs, 1990).

In tumors of the epidermis, this spatially and temporally ordered program of differentiation is disrupted. The inappropriate expression of K13 in mouse papillomas has been correlated with their malignant potential (Conti et al., 1990; Sutter et al., 1991). Most carcinomas of the epidermis express the simple epithelial-specific keratins K8 and K18 (Guerra et al., 1992; Cheng et al., 1990). Furthermore while a subset of cells in carcinomas express the keratin K1/K10 pair, most carcinoma cell lines in vitro fail to express this pair (Yuspa, 1987).

Keratin gene expression is very useful in assigning the differentiated state of a given keratinocyte cell or tumor. The work presented in latter chapters demonstrates that escape from differentiation controls is intrinsically involved in tumor progression. Furthermore, work presented demonstrates that tumor suppressor genes may suppress tumorigenicity by inducing differentiation programs.

**Tumor Suppressor Gene Inactivation**

The oncogene hypothesis greatly changed mechanistic views of carcinogenesis. This hypothesis directly addressed the question of causation for cancers of environmental origin. Retroviruses were known to carry activated homologs of proto-oncogenes (Bishop, 1987; Bishop, 1991). Chemical and physical carcinogens were assumed to activate similar proto-oncogenes (Duesberg, 1985; Balmain and Pragnell, 1983; Balmain et al.,
Today the list of oncogenes nears 100; however, the oncogene hypothesis does not appear to be a grand unifying theory of carcinogenesis. This hypothesis does not appear to address genetic predisposition to cancer. Families and physicians have known for centuries that cancers ran in families suggesting a heritable component of cancer causation. The multistage model of carcinogenesis and the oncogene hypothesis address only exogenous initiators of tumorigenicity. The scientists in carcinogenesis needed a theory to help explain genetic predisposition to cancer. This need was partially addressed by the proposal of the tumor suppressor gene hypothesis.

The brief history of tumor suppressor genes began in 1969 when Henry Harris demonstrated the suppression of malignancy in somatic cell hybrids (Harris et al., 1969). In contrast to an earlier report, these authors showed that in somatic cell hybrids, malignancy was the recessive phenotype. Because at this time it was so generally and widely accepted that malignancy was a dominant phenotype, these authors speculated little as to the cause of this controversial finding. In a nearly self-deprecatory final paragraph, they stated that their results could be due to the azaguanine resistance of the normal cell or perhaps just an odd artefact of that cell line alone. However, in response to public challenges as to the validity of their results, these authors noted that tumorigenic segregants carried fewer chromosomes than their suppressed counterparts. Malignancy, they hypothesized may be the result of chromosome loss. Two years later, in 1971, Alfred Knudsen while studying rare childhood retinoblastomas, proposed that statistical analysis of tumor incidence suggested a multi-hit hypothesis (Knudsen, 1971). More
simply, he proposed two genomic lesions or "hits" were required for initiation of carcinogenesis. Retinoblastoma was known to be both familial, that is clustering in similar genetic backgrounds, and sporadic. Familial tumors had an earlier age at onset than sporadic cases and were frequently bilateral. He reasoned that in familial retinoblastoma children inherited a damaged allele, and the second event was due to a somatic mutation. In contrast, he argued that sporadic cases were the result of two somatic mutations. A possible explanation for the hereditary basis of tumor formation had been proposed.

The term tumor suppressor gene seems to have evolved slowly. While Harris and Klein first discussed the "suppression of malignancy," Knudsen championed the term anti-oncogene in response to the popularity of oncogenes (Klein, 1987; Klein, 1988). Some researchers favored the term recessive cancer genes, and George Todaro suggested emerogene, Latin for to tame oncogenes (Klein, 1987; Klein, 1988). However, as the mechanism and function of these unknown genes became more clear, the term tumor suppressor gene became generally accepted. Like the similar problems the earlier names had, the term tumor suppressor gene itself is likely to be a misnomer. As recent evidence indicates, these genes are more likely to be involved in cell cycle control and not tumor suppression per se. The role and concept of tumor suppressor genes will be discussed in a latter chapter.

Knudsen's hypothesis was elegantly substantiated in 1983 when karyotypical analysis located Knudsen's "hits." (Benedict et al., 1983; Godbout et al., 1983; Cavenee et al.,
In 1983, the frequent identification of interstitial deletions in band q14 of chromosome 13 led to the coining of the term RB-gene. The search was on for isolation and identification of the first known tumor suppressor gene. The RB-1 gene was isolated and cloned by three different groups in 1987 (Friend et al., 1987; Fung et al., 1987; Lee et al., 1987). In the six years since, research has indicated that the Rb protein is a phosphoprotein intricately involved in the regulation of cell cycle progression (Sager, 1992). Very recent results implicate CDC2-like kinase-cyclin complexes in the phosphorylation of Rb (Tsai et al., 1991). Furthermore, Rb apparently interacts directly with adenovirus gene products and may modulate the activity of the transcription factor E2-F (Chellappan et al., 1991).

The tumor suppressor field was shocked in 1989 when Michael Green suggested that widely accepted oncogene p53 might actually be a tumor suppressor gene (Green 1989). p53 was initially identified as a protein bound to large T antigen in SV40 transformed cells. Initial studies indicated that p53 could transform non-tumorigenic rodent cells (Green, 1989; Finlay et al., 1989). This apparent contradiction was resolved when it was discovered that the initial studies were conducted with a mutated p53 (Kern et al., 1991; Friedman et al., 1990). Evidence today indicates that wild type p53 can actually suppress transformation. Support for its role as a tumor suppressor gene are threefold: 1) p53 is mutated or lost in very large number of tumors and transformed cell lines, 2) p53 binds viral oncoproteins, and 3) wild type p53 elicits growth inhibition in transformed cells. Like Rb, p53 seems to be elaborately involved in cell cycle regulation. p53 is a known
DNA binding protein and appears to have transactivation activity (Kern et al., 1991; Friedman et al., 1990). Recent reports indicate that wild type p53 may be directly involved in inhibiting the initiation of replication (Kern et al., 1991; Friedman et al., 1990). Consistent with this hypothesis is the observation that p53 accumulates in cytoplasm during G1 phase of the cell cycle, and then it enters the nucleus at G1/S boundary (Shaulsky et al., 1990). Significantly like Rb, p53 is also phosphorylated by a CDC2-cyclin complex. The interaction between cyclins, p34CDC2 and tumor suppressor genes may ultimately determine how and when cell cycle progression occurs.

Today nearly ten putative tumor suppressor genes have been characterized, and the search appears to be far from saturated (Marx, 1993). Unlike oncogenes, however, the very nature of tumor suppressor genes makes their characterization difficult. In fact, the very existence of tumor suppressor genes is often postulated by their absence. This apparent paradox makes them difficult to study. DNA-mediated gene transfer experiments greatly facilitated the study of oncogenes and helped promote the term dominant acting transforming activity. However, unlike oncogenes, no easy experimental assays exist for their identification. The bulk of the reason for this is the extreme difficulty in selecting single non-transformed clones in a background of rapidly proliferating tumorigenic cells. The apparent paradox of the genetic dominance of oncogenes and the role of natural selection in establishment of a malignant phenotype is the main thesis of the work presented here. The nature and mechanism of tumor suppression will be analyzed and discussed in the context of in vivo and in vitro carcinogenesis.
Cancer in the 1990's: The Status Quo

Cancer research in the 1990's has been very promising. The tumor suppressor genes, Rb and p53, have been established to have roles in cell cycle progression (Sager, 1992). Three potential tumor suppressor genes involved in colorectal carcinogenesis have been identified, DCC, MCC, and FAP (Marx, 1993; Vogelstein, 1993). Two other potential tumor suppressors have been implicated in Neurofibromatosis. NF-1 and NF-2, and the Wilm's tumor locus has been cloned and identified at WT-1 (Marx, 1993; Kinzler and Vogelstein, 1993). Heterodimeric partners of myc have been identified in the genes max and mad, and the mechanism of myc transactivation is becoming more clear (Blackwood and Eisenman, 1991). The role and origin of metalloprotease expression in metastasis has shifted in focus from the tumor cell to an interaction between the surrounding stroma and tumors (Muller et al., 1993). After a decade of searching, the signal transduction pathway of the Ras oncogene to the nucleus is beginning to be understood (Marx, 1993; Blennis, 1993). The implication of the Bcl2 oncogene, apoptosis and cancer appears to be a promising angle of research (Marx, 1993). Finally the discovery that the Prad1 oncogene is a mammalian cyclin homolog may help bridge the gap between carcinogenesis and cell cycle regulation.

The horizon is bright, and a great many discoveries are surely just around the next corner. However, in the meantime new questions need to be asked, new hypotheses proposed, and old theories reconsidered. Most importantly the last decade of
observational research needs to be transformed into mechanistic theories that not only explain existing data but make significant predictions about discoveries yet to be made. Specifically, new observations need to be integrated into the multistage theory of carcinogenesis. A realistic and testable number of steps and stages in the model need to be defined. The role and temporal significance of oncogene activation and tumor suppressor gene inactivation needs to be further clarified. New mechanisms of action need to be put forward to explain the role for all genes and gene products associated with carcinogenesis. New technology needs to be accessed in order to test or disprove lingering old theories. Carcinogenesis research is in a period of explosive growth, and the time has come to consolidate observations, hypotheses, and results into a unified, and most importantly, testable theory that not only explains the tumor cell but also leads to a greater insight into the inner workings of the wild type cell.

The Model System

The long latency of human tumors and the difficulty of transforming human cell lines has necessitated the investigation of rodent models of carcinogenesis. As stated previously, the model utilized for this work was designed to simulate human epithelial carcinogenesis. The importance of such a model is underscored by the realization that 78% of new cancers and 73% of deaths in 1993 will be from cancers of the epithelia. This model utilizes nontumorigenic murine keratinocyte cell line, 291, as the progenitor
of tumorigenic lines. Initiation in vitro with the chemical carcinogen 7,12-dimethylbenz[a]anthracene generated tumorigenic cell lines. Subsequently clonal cell lines were derived representative of distinct stages in the multistage model of carcinogenesis. One cell line, 09, forms benign papillomas upon transplantation onto the backs of nude mice. Another cell line, 03, forms malignant highly invasive carcinomas upon either transplantation or subcutaneous injection. These tumors are similar to human squamous cell carcinomas of the head and neck.

Statement of the Problem

The objective of this research was to analyze an in vitro mouse model of epidermal carcinogenesis in the context of human cancer. Three independent hypotheses were investigated. First, it was hypothesized that a balance between genetic alterations and selective pressures determines the genotype of a given cancer cell. Specifically the frequency and role of ras activation was addressed. Second, I hypothesized that the progression of a nontumorigenic cell through a benign state to frank malignancy requires the loss of multiple tumor suppressor genes. Somatic cell hybrid studies were utilized to address this question. Finally, it was hypothesized the identification of genes expressed exclusively in either the tumorigenic or nontumorigenic cell lines. Differential northern analysis and subtractive hybridization were utilized to investigate this hypothesis.
Specific Aims of This Dissertation

The research presented here is a both a result of and a continuation of projects ongoing in Dr. Molly Kulesz-Martin's and Dr. Tim Bowden's laboratories. In undertaking this research, three specific aims were addressed. First, *in vivo* and *in vitro* models of carcinogenesis were compared. Specifically the relationship between chemical initiation and oncogene activation were investigated and the role of natural selection in the propagation of a tumor's genotype is discussed. Second, the expression of keratin genes as markers of tumorigenicity was investigated. In addition, subtractive hybridization identified 5 putative gene sequences (SUZ 1-4 and 6) which were expressed exclusively only in the non-tumorigenic mouse keratinocyte cell line. Finally, somatic cell hybrids were constructed to analyze the role of tumor suppressor gene loss in carcinogenesis. These studies indicated that induction of differentiation was a principal means of tumor suppression. The objective of this research was to analyze a particular mouse model of carcinogenesis in the context of its applicability to human carcinogenesis.

Chapter six of this dissertation addresses the specificity of chemical initiation in either *in vitro* or *in vivo* models of carcinogenesis. DNA-mediated gene transfer experiments indicated a transferrable dominant transforming activity in NIH3T3 fibroblasts. However, mutated Ha-*ras* alleles were not detected in the tumorigenic mouse cell lines studied despite the direct correlation between chemical initiation and *ras* activation *in vivo*. The role of natural selective pressures and promotion are discussed
in the context of these results.

The dominant or recessive nature of malignancy in somatic cell hybrids is examined in chapter seven. These studies demonstrated the loss of multiple tumor suppressor activities in the progression of tumorigenicity in this model. All of the hybrids created showed varying degrees of tumor suppression. The nontumorigenic cells suppressed tumor formation to a greater extent than the benign cells. Interestingly an independently derived less malignant well differentiated carcinoma cell line was also able to suppress tumor formation. Like the parental nontumorigenic cells, the somatic cell hybrids also express the keratin gene Kl in response to high calcium (1.4 mM) in vitro. In addition, all of the hybrids had fewer local metastases, and when tumor formation was not suppressed, the resulting tumors tended to be highly differentiated. Furthermore, PCR analysis of injection sites that failed to form tumors indicated an absence of injected hybrids. These data are consistent with the hypothesis that tumor suppression is occurring through induction of differentiation. The demonstration that a benign papilloma forming cell line can suppress malignancy has important implications and role and timing of tumor suppressor gene loss in carcinogenesis, and the suppression of papilloma formation by a nontumorigenic epidermal cell is a novel finding.

Evidence that the tumorigenic cell lines studied here possess differentiation defects is presented in chapter eight. Analysis of keratin gene expression in these mouse cell lines confirmed the putatively initiated phenotype of the papilloma line. Furthermore, unlike their non-tumorigenic progenitor, the tumorigenic cell lines failed to express either
keratin K1 or K10 in response to high extracellular calcium concentrations indicative of an inability to differentiate. While differential northern analysis revealed that keratins K6 and K18 were predominately expressed by either the non-tumorigenic or malignant cells, respectively, subtractive hybridization identified five putative gene sequences (SUZ 1-4 and 6) expressed exclusively by the non-tumorigenic cell line. SUZ4, the most extensively studied, was also not expressed in eight other tumorigenic cell lines examined. This putative gene was highly homologous to a non-coding region of lactate dehydrogenase, and hybridized strongly to a small transcript in murine hearts.

In summary, a rodent model of carcinogenesis was examined for its adaptability to human carcinogenesis. The specificity of chemical initiators was examined. The role of natural selection in both endogenous and exogenous promotion was considered. The demonstration of a differentiation defect in tumorigenic cells that was complemented in somatic cell hybrids led to a general discussion of the potential dominant or recessive nature of oncogenes and tumor suppressor genes. The evidence that multiple tumor suppressor genes may act at discrete stages in epithelial carcinogenesis may aid in a greater understanding of carcinogenesis and lead to more successful treatment of cancer.
VI. MATERIALS AND METHODS

Tissue Culture

Cell Lines

Keratinocyte cell line was derived from neo-natal Balb/c Ros epidermis according to a modified procedure for isolating primary epidermal cells (Kulesz-Martin et al., 1985; Yuspa et al., 1981). As previously reported, 291 cells were chemically initiated in vitro with 0.2-0.4 mM DMBA for 24 hours (Kulesz-Martin et al., 1988). Putatively initiated clones 09, 03, and 05 were selected for by their ability to escape terminal differentiation induced by increased extracellular calcium concentrations. Each clone was independently derived and represents a discrete stage in multistage carcinogenesis: 291, nontumorigenic epidermis; 09 benign papillomas; 05, well differentiated carcinomas; and 03, undifferentiated locally metastatic carcinomas.

Culture Medium and Standard Culture Technique

Eagle’s minimum essential medium (EMEM) without CaCl₂ (Gibco, Grand Island, NY) is used to prepare a low calcium essential medium (LCEM). Chelex 100 resin
(BioRad Laboratories, Rockville Center, Long Island, NY) is used to reduce Ca\(^{2+}\) in Fetal bovine serum (FBS) (Gibco). Eight hundred grams of resin are resuspended in distilled water, and the pH is adjusted to 7.5. Drained and filtered resin is then mixed with 1 liter of FBS and stirred for one hour at room temperature. Chelex resin is removed by filtering through 3 mm Whatman paper, and the FBS is sterilized by filtering through .22 micron filters. The calcium concentration of chelexed serum is determined by atomic absorption spectroscopy. Primary epidermal cultures and the cell lines utilized here require unidentified growth factors secreted by dermal fibroblasts. These factors are obtained by conditioning LCEM for 48-72 hours on cultures of dermal fibroblasts (Kulesz-Martin et al., 1984; Yuspa et al., 1980). The resulting conditioned media is referred to as conditioned low calcium Eagle's minimum essential medium (CLCEM). LCEM is prepared to the following specifications: 5% chelexed FBS, 5% CLCEM, 0.8% antibiotic/antimycotic solution (Gibco), 10 ng/ml epidermal growth factor (EGF), and the final Ca\(^{2+}\) is adjusted to 0.04 mM. High calcium essential medium (HCEM) is prepared by adding 10.3 ml of 10 g/L CaCl\(_2\) to 500 ml of LCEM for a final Ca\(^{2+}\) concentration of 1.4 mM.

**Calcium Phosphate Mediated Gene Transfection for Generation of Drug Resistant Cell Lines**

All cell lines were plated at approximately 1 x 10\(^6\) cells per 100 mm dish.
Transfection assays were performed by the calcium phosphate co-precipitation method (Wigler et al., 1978). Five micrograms of either pKCR3hygro or pSV2neo were placed on each 100 mm plate. Sixteen to twenty four hours later, each plate was washed with serum free media. After 48 hours in serum containing media, clones were selected for in the presence of 400 μg/ml G418 and 100 μg/ml Hygromycin. Single clones were picked and rapidly expanded.

DNA Transfections for the Detection of Dominant Transforming Activity

Low passage (P12-14) NIH3T3 fibroblasts were used as the recipient of genomic DNA isolated as previously described (Bonham et al., 1989) from non-tumorigenic, benign, or malignant cell lines. NIH3T3 cultures were seeded at a density of 1 x 10^6 cells/plate 24 hours prior to transfection. Transfection assays were performed by the calcium phosphate co-precipitation method (Wigler et al., 1978). Mock transfections containing no DNA and NIH3T3 DNA into NIH3T3 cells served as negative controls. CarB genomic DNA (Buchmann, et al., 1991), which contains an activated c-Ha-ras oncogene, served as the positive control. Eighteen hours after transfection, plates were trypsinized and replated at 25% their original density. Cultures were maintained in DMEM containing 5% calf serum, and plates were scored for morphological transformation 21-28 days after transfection.

Polyethylene Glycol Cell Fusion For the Generation of Somatic Cell Hybrids
A mixture of $1 \times 10^6$ cells of each parental line was plated on 60 mm plates twenty four hours before cell fusion. Five grams of PEG MW 3,000-3,700 was autoclaved for 5 min at $121^\circ$ C. Five ml of 2x sterile tissue culture medium without serum and pre-warmed to $37^\circ$ C was added to the cooling PEG to prepare a 50% solution. One ml was added slowly to the cocultured cells and incubated for 1 min at $37^\circ$ C. One ml serum free medium was added and incubated another minute. In consecutive steps, two then four mls of serum free medium were added for two and four minutes, respectively. Serum containing medium was then added to each plate and incubated 48 hours at $37^\circ$ C. After two days, each 60 mm plate was trypsinized and replated onto four 100 mm plates for selection.

**Subcloning**

Clonal cell lines from each somatic cell hybridization were generated by either ring cloning or limiting serial dilutions. When possible, individual colonies were ring cloned into 16 well plates. Each cell was then rapidly expanded and cryopreserved. When individual colonies could not be ring cloned, limiting serial dilutions were plated in 96 well plates. Single wells were then trypsinized, expanded, and cryopreserved as above.

**Calcium Inductions**
A stock bottle of 10 g/liter CaCl₂·7H₂O was used to prepare media containing low (0.04 mM), medium (0.5 mM), or high (1.4 mM) calcium concentrations. Calcium inductions were conducted by removing standard tissue culture medium, washing with calcium free MEM, and replacing with either low, medium, or high calcium containing medium. Cells were then incubated for 24-48 hours before protein and RNAs were harvested.

**Nude Mouse Tumorigenicity and Histology**

Parental and hybrid cells were grown to approximately 90% confluence before harvesting. Cells were trypsinized, counted, and resuspended at a concentration of 1-2 x 10⁶ cells per 0.2 ml calcium free MEM. Sub-cutaneous injections were performed on all four flanks of female nu/nu mice with a Balb/c background from Harlan Sprague Dawley, Indianapolis, IN. Skin grafts of hybrids or parental cells were performed essentially as previously described (Kulesz-Martin *et al.*, 1988). Animals were monitored twice a week, and tumor growth was assessed by measuring the largest diameter using calipers. Tumorigenicity was initially assessed on the appearance of a visible and progressive nodule, and confirmed by pathology and histology. Mice were observed for up to four months. When tumors were 5 to 12 mm in diameter, they were harvested, fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histology.
Polymerase Chain Reaction

Detection of Neo Gene Transcripts

Total genomic DNA was isolated from confluent 100 mm culture dishes of each cell line as previously described (Bonham et al., 1989). PCR primers, designed to amplify a band corresponding to part of the coding region of the Neomycin resistance gene, were synthesized on an Applied biosystems 391 DNA synthesizer. Amplification conditions were as follows: a 20 µl reaction mixture contained 1 µg of genomic DNA, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.1 mg/ml gelatin, 1 µM of each primer, 200 µM dNTPs, and 2.5 units of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN). Each reaction mixture was overlaid with 20 µl of mineral oil and the DNA was denatured at 94°C for 5 min. Amplification was performed for 20 cycles with 1 min at 94°C for denaturation, 2 min at 60°C for annealing, and 3 min at 72°C for primer extension. PCR products were analyzed by 1.5% agarose gel electrophoresis.

PCR of c-Ha-ras and Xba I Restriction Analysis

Total genomic DNA was isolated from confluent 100 mm culture dishes of each cell line as previously described (Bonham et al., 1989). PCR primers(29-mers, US-RAS 5'GAGAGAATTCCTGCTGAGCTATGAC3', DS-RAS 5'CACAGAATTCACGG-
AAGCTTCCCTACG 3') for the amplification of a 588 bp band corresponding to exons one and two of the genomic c-Ha-ras gene were synthesized on an Applied Biosystems 391 DNA synthesizer. Amplification conditions were as follows: a 20 µl reaction mixture contained 1 µg of genomic DNA, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.1 mg/ml gelatin, 1 µM of each primer, 200 µM dNTPs, and 5 units of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN). Each reaction mixture was overlaid with 20 µl of mineral oil and DNA was denatured at 95°C for 5 min. Amplification was performed for 30 cycles with 1 min at 95°C for denaturation, 1 min at 63°C for annealing, and 1 min at 72°C for primer extension. Following amplification, the reaction mixture was extracted once with chloroform, and 5 µl were transferred to a 20 µl digestion reaction for Xba I restriction. PCR products were analyzed by 1.5% agarose gel electrophoresis. Restriction fragments obtained by cleavage of pBR322 with either Bst NI or Msp I were used as size standards.

**PCR of c-Ha-ras and Discriminative Oligonucleotide Dot Blot Hybridization**

Total genomic DNA was isolated from a confluent 60-mm culture dish of each cell line as described (Blin et al., 1976). PCR primers for the amplification of murine Ha-ras codon 61 or 12 and 13 were obtained from Clontech Laboratories (Palo Alto, CA) or provided by M. Schwarz (Heidelberg, FRG). Amplification conditions were chosen as follows: A 100-µl reaction mixture contained 1 µg of genomic DNA in 10 mM Tris-HCl,
pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, 0.001% gelatin, 1 µM of each primer, and 200 µM dNTPs. Reaction mixtures were overlaid with 90 µl mineral oil, and denatured at 95°C for 5 min. Subsequently, 2 units of Taq polymerase (Perkin Elmer/Cetus) were added, and the primers were allowed to anneal at 45°C. Thermal cycling was performed for 35 rounds with 1.0 min at 95°C for denaturation, 1.0 min at 45°C for primer annealing, and 1.5 min at 72°C for primer extension. PCR products were analyzed by 2% agarose gel electrophoresis with size markers (restriction fragments obtained by cleavage of ØX174-DNA with Hae III).

Equal amounts of the amplified DNAs were spotted onto nylon membranes (Biotrace RP, Gelman) and the DNA was fixed by baking for 2 h at 80°C. Membranes were prehybridized for 4 h at 50°C in a mixture of 5X SSPE, 0.3% SDS, and 500 mg sonicated herring sperm DNA and subsequently hybridized for 18 h in the same mixture with either the $^{32}$P-end-labeled 20-mer oligonucleotide for the wild type sequence or the 20-mers modified in base 1, 2, or 3 of codons 12, 13, or 61 (Brown et al., 1990). After hybridization, the membranes were washed twice in 2X SSPE and 0.1% SDS for 30 min at room temperature followed by stringent washes in 5X SSPE and 0.1% SDS for 10 min at selective discriminating temperatures (58°-68°C). Subsequently the membranes were exposed to Kodak X-Omat films at -80°C using intensifying screens.

**Southern Analysis**
Twenty micrograms of genomic DNA from keratinocyte cell lines or NIH3T3 cell primary transfectants were digested with Eco RI, resolved by 1% agarose gel electrophoresis, and transferred to GeneScreen nylon membranes (New England Nuclear, Boston MA) according to manufacturer's instructions. DNA was UV-linked to the membranes using a Stratalinker (Stratagene, La Jolla, CA). Membranes were incubated overnight at 42°C in a solution of 50% formamide, 10 X Denhardt's solution, 50 mM Tris-HCl (pH 7.5), 0.1% sodium pyrophosphate, 1% SDS, 1 M NaCl, 10% dextran sulfate and 200 mg/ml DNA from salmon testes. Random priming (US Biochemicals, Cleveland, OH) was used to $^{32}$P-label a c-Ha-ras (a 600 bp Eco RI - Bam HI fragment of p65GE9EB2 (Bonham et al., 1989)) probe to a final specific activity of 2-4 x $10^6$ cpms/ml. After 24 hours of hybridization at 42°C, membranes were washed twice with 2X SSC at room temperature for 5 min, twice with 2X SSC and 1% SDS at 65°C for 30 min, and twice with 0.1X SSC at room temperature for 30 min. Membranes were exposed to Kodak X-Omat films at -80°C using intensifying screens.

**Northern Analysis of c-Ha-ras**

Cells were harvested when approximately 70-100% confluent. RNA was isolated by guanidinium/cesium chloride extraction and dissolved in diethylpyrocarbonate-treated water for northern blot analysis as described previously (Han et al., 1990). Ten micrograms of total cellular RNA were resolved by 1.2% agarose gel electrophoresis, transferred to a
nylon membrane, and hybridized with a $^{32}$P-labeled Ha-ras probe (830 bp Hind III fragment of Ha-MuSv (25)). An 840 bp Eco RI-Sal I fragment of pA6 (26) was used for 7S RNA detection which served as an internal control for RNA loading. Probes were labeled with $^{32}$P dTTP by the random primer method using a multiprime DNA labeling kit (Amersham, Arlington Heights, IL). $^{32}$P-labeled probe was used at the final concentration of 1 to 2 x $10^6$ cpm/ml. Quantitation of relative amounts of RNA was done by densitometry of exposed films using a Quick Scan (Helena Laboratory, Beaumont, TX) and Fastscan computing densitometer (Molecular Dynamics, Sunnyvale, CA). The c-Ha-ras hybridization signals were compared after adjustment for differences in 7S RNA.

Other Northern Analyses

Total RNAs were isolated from sub-confluent plates of each cell line as previously described (Chomczynski and Sacchi, 1987). Each cell line was grown according to the conditions reported in each Figure legend. Fifteen micrograms of total RNA were resolved by 1% agarose gel electrophoresis. RNAs were transferred to Genescreen as per manufactures instructions. Each blot was subsequently reprobed with a 7S or GAP-(Glyceraldehyde 3-phosphate dehydrogenase)-specific control to assure equal RNA loading and transfer.

Subtractive Hybridizations
Specific PCR amplification of \( \lambda \) cDNA Inserts

2-10 \( \mu l \) of a directional library was used in a 20-50 \( \mu l \) PCR reaction. Each PCR reaction contained: 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl\(_2\), 50 mM KCl, 0.1 mg/ml gelatin, 100 pmol of T3 and oligo dT/Eco RI primers (synthesized on an Applied Biosystems oligonucleotide synthesizer), 200 \( \mu M \) dNTPs, 0.5 ml \(^{32}\)P-dCTP (NEN, 3000 Ci /mmol, 10 mCi /ml), and 5 units of Taq polymerase (Boehringer Mannheim, Indianapolis, IN). The cycling conditions were as follows: 1 cycle of 3 min of denaturation at 94\(^\circ\)C, 2 min of annealing at 50\(^\circ\)C, and 7 min of polymerization at 72\(^\circ\)C; 38 cycles of 1 min at 94\(^\circ\) C, 2 min at 50\(^\circ\)C, and 9 min at 72\(^\circ\)C. Aliquots of individual PCR reactions were analyzed by 1.5% agarose gel electrophoresis.

Oligo dT-Cellulose selection of cDNA Inserts

PCR reactions were extracted with an equal volume of chloroform to remove mineral oil. Final sample volumes were adjusted to 100 \( \mu l \) and 1 \( \mu l \) was counted in scintillation fluor. The remaining sample was washed by the addition of 2 ml of 0.1% SDS and centrifugation through a pre-washed centricon-30 (Amicon) at 5000 x g for 15 min. The cDNA was washed twice more as described above and the recovered volume was adjusted to 100 \( \mu l \). 1\( \mu l \) was removed for quantitation.
To isolate single stranded DNA from the amplified cDNAs, oligo dT-cellulose (New England Biolabs, Beverly, MA) columns were poured as previously described (Maniatis et al., 1982). Double stranded cDNAs were boiled in 20 mM Tris-HCl pH 7.6, 0.5 M NaCl, and 1 mM EDTA, loaded onto the column, and washed twice in loading buffer. Eluate fractions (target sequences) were collected, boiled and re-applied to the column. Scintillation counting approximated the percentage of recovered single stranded poly dT cDNAs. Single stranded poly dA sequences (driver sequences) were eluted from the column with 2-4 volumes of 10 mM Tris-HCl pH 7.5 and 1 mM EDTA.

**Preparation of Biotinylated Driver mRNA**

Previous experimentation indicated that a fifteen molar excess of biotinylated driver was sufficient to subtract 98-99% of common sequences when driver was self-hybridized. To biotinylate the driver, poly dA single stranded sequences were precipitated, resuspended in ddH$_2$O at a final concentration of 1 µg/µl and mixed with an equal volume of 1 mg/ml Photoprobe biotin (VectorLabs, Burlingame, CA). Mixtures were irradiated on ice for 15 min with a Sunlamp (VectorLabs, Burlingame, CA) 10 cm from the open tube. After labeling, the volume was adjusted to 100 µl with 0.1 M Tris-HCl, pH 9.5, and extracted twice with 2-butanol. Biotinylation was repeated to saturate the number of biotinylated residues. Biotinylated single stranded poly dA sequences were then precipitated and quantitated.
**Subtractive Hybridization**

0.1-1 μg of $^{32}$P-labeled single stranded poly dT cDNAs were precipitated with a 15-fold molar excess of biotinylated single stranded poly dA driver sequences. Precipitates were pelleted by microcentrifugation at maximum speed for 1 hr. Pellets were washed with 80% ethanol, Chernkov counted, then resuspended in 2 μl of 100 mM Hepes, pH 7.6, 0.4% SDS, and 4 mM EDTA. After resuspension, 2 μl of 1 M NaCl saturated with dextran blue was added (for visualization of the aqueous phase). The solution was overlaid with 50 ml of mineral oil, boiled for 2 min, and transferred to a 68°C waterbath for 24-48 hours until a $R_{o,t}$ of greater than 4500 was achieved. The 4 μl hybridization reaction was added to 96 μl of 150 mM Tris-HCl, pH 7.5, and 150 mM NaCl. For removal of biotinylated sequences Vectrex avidin (VectorLabs, Burlingame, CA) was prepared by resuspension in the same buffer at a final concentration of 160 μg/μl. 100 μl of Vectrex avidin were added to each hybridization reaction and gently vortexed for 5 min. Common double stranded sequences and excess biotinylated driver were removed by microcentrifugation at maximum speed for 5 min. The aqueous phase was removed and extracted with phenol/chloroform. Extraction of biotinylated sequences with avidin was repeated prior to quantitation.

**PCR Amplification of Subtracted cDNAs**
Subtracted single stranded cDNAs were precipitated and resuspended in 20 µl of ddH₂O. 2-5 µl of subtracted cDNAs were PCR amplified in the following PCR reaction mixture: 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.1 mg/ml gelatin, 100 pMol of T3 and oligo dT/Eco RI primers (synthesized on an Applied Biosystems oligonucleotide synthesizer), 200 µM dNTPs, 0.5 µl ³²P-dCTP (NEN), and 5 units Taq polymerase (Boehringer Mannheim). The cycling conditions were as follows: 1 cycle of 3 min of denaturation at 94°C, 2 min of annealing at 50°C, and 7 min of polymerization at 72°C; 38 cycles of 1 min at 94°C, 2 min at 50°C, and 9 min at 72°C. Aliquots of individual PCR reactions were analyzed by 1.5% agarose gel electrophoresis.

**Generation of Subtracted Probes**

PCR reaction mixtures were centrifuged in a Centicon-100 column as described above and extracted with phenol/chloroform. Resulting supernatants were precipitated, resuspended in ddH₂O, and quantitated. Subtracted probes were generated by using 500 ng of subtracted PCR product in a standard random prime procedure (Maniatis et al., 1982; Ausubel et al., 1992). Oligo dT primers were used in place of random primers, but all other conditions were as previously described (Maniatis et al., 1982; Ausubel et al., 1992). Subtracted probes were used to screen non-tumorigenic 291 libraries. Putatively positive clones were picked and suspended in 0.5 ml phage dilution buffer. Inserts were amplified using the PCR procedure described above. Individual plasmids were rescued from the
Lambda ZapII vectors as described by the manufacturer. Plasmids containing inserts were tested with plus/minus northern screening of spaghetti blots containing nontumorigenic, 291, benign, 09, or malignant, 03 total RNAs. Those inserts hybridizing exclusively to only the nontumorigenic, 291, RNAs were selected and sequenced.

**Misc. Molecular Biology Techniques**

All techniques not specifically delineated are standard molecular biology techniques as previously described (Maniatis et al., 1982; Ausubel et al., 1992).
The Model System and Hypotheses Addressed

While the activation of the ras family of genes is an example of a genetic change frequently associated with carcinogenesis, considerable inconsistencies exist concerning the necessity and role of ras activation in carcinogenesis. In the context of examining the driving force behind the perpetuation and selection of genetic changes responsible for carcinogenesis, I propose that inconsistencies in ras activation frequencies in different animal and cell culture models are a result of different local environmental selective pressures. While the evidence for the role of selection in the origin and propagation of mutations lies in bacterial model systems (Luria and Delbruck, 1943; Cairns et al., 1988), extrapolation of these observations may further elucidate the mechanism and role of selective pressures on ras mutation frequencies in models of carcinogenesis. Specifically, I propose that the extracellular environment and the temporal order of ras activation with regard to other genetic changes result in either the propagation of or selection against cells containing ras mutations. In the simplest terms, when ras activation is involved in carcinogenesis, ras mutations could be selected for by their ability to contribute phenotypic changes advantageous to the tumorigenic cell, and selected against when the
genetic cause of the favorable phenotype is not a direct result of ras activation.

The data presented here demonstrate that cloned mouse cells initiated with DMBA in vitro generate benign and malignant tumorigenic phenotypes very similar to human squamous cell tumors. In addition, they are indistinguishable from mouse epidermal tumors produced during in vivo two-stage carcinogenesis with DMBA and 12-O-tetradecanoylphorbol-13-acetate (TPA) treatments. Because there is no evidence for activation of the Ha-ras proto-oncogene, this cloned keratinocyte model may be useful for the identification of gene targets more pertinent to human skin carcinogenesis, as well as for functional studies of ras gene activation at discrete stages of carcinogenesis.

These results fuel speculation that the adaptability of an initiated cell in its interaction with its immediate environment may ultimately determine the genotype of the resultant tumor. I propose that exogenous tumor promotion accentuates the selective pressures of a given environment, and that it is the interaction of the tumor cell with its locale that determines the spectrum of activated oncogenes detected in the tumor and not the initiating carcinogen.

Introduction

The process of experimental induction of animal tumors can be operationally subdivided into several stages (Boutwell, 1974). While each stage is likely to be the result of a number of genetic changes, the timing and function of these genetic changes
are incompletely defined. The multistage model of carcinogenesis defines steps in tumor formation, but without genetic cause-and-effect relationships, the model is merely a theoretical definition. A wide variety of cell-culture and animal model systems have been developed in an attempt to elucidate the genetic nature of these cause-and-effect changes. While both the necessity and sufficiency of oncogene activation in tumor formation have been questioned, most of this speculation concerns the effects of the genetic changes. In addition, the causes of genetic changes responsible for tumor formation are under constant investigation. However, what may perhaps be more important is the mechanism of perpetuation and selection of these genetic changes. Initiation events occur more frequently than can be accurately estimated by tumor formation frequencies (Gould et al., 1987). The mechanism whereby genetic changes are either propagated or lost may contribute more to the process of tumor formation than the initial cause of the genetic change does. While the activation of the ras family of genes is an example of a genetic change frequently associated with carcinogenesis, considerable inconsistencies exist concerning the necessity and role of ras activation in carcinogenesis.

The ras Family of Oncogenes

In attempting to understand the molecular basis of cancer causation, scientists first discovered ras oncogenes in the acutely transforming Harvey and Kirsten rat sarcoma viruses (Barbacid, 1987). The name ras itself is an acronym for rat sarcoma. Gene
transfer studies for the identification of dominant transforming activity in human and animal tumors led to the inadvertent rediscovery of ras oncogenes (Shih et al., 1981; Krontiris et al., 1981; Perucho et al., 1981; Pulciani et al., 1982; Sukumar et al., 1983). The discovery that human and animal tumors possessed mutated viral oncogene homologs greatly strengthened the view that oncogene activation was responsible for carcinogenesis. Since their original discovery, the ras family of oncogenes has found to have three members, Ha-ras, K-ras, and N-ras, which are 85% homologous to one another (Barbacid, 1987). Evolutionarily, ras genes are extremely well conserved. The yeast ras gene product and the mammalian gene product have been shown to be interchangeable (DeFeo-Jones et al., 1985; Katakoa, et al., 1985), and recent evidence suggests the existence of a super gene family.

The ras gene family codes for proteins of a relative molecular weight 21,000 and are referred to as p21 proteins. These p21 proteins bind guanine nucleotides and have an intrinsic GTPase activity (Barbacid, 1987). Like the two other families of GTP binding proteins, the heterotrimeric G proteins and elongation and initiation factors of protein synthesis, p21rar is biologically active while in the GTP bound state (Gilman, 1987; Thompson, 1988; McCormick, 1989; Downward, 1992). Hydrolysis of GTP to GDP results in an inactive form. In 1987, a stimulator of the intrinsic GTPase activity was identified in Xenopus oocyte extracts (Trahey and McCormick. 1987). This protein, a 120,000 MW protein, was named GAP for GTPase activating protein. GAP is a negative regulator of p21rar, but is ineffective against mutated ras oncogenes.
The powerful transforming activity of ras in vitro has focused research on the mutational activation of this oncogene. While point mutations have been identified in eight different codons, 12, 13, 59, 61, 63, 117, 119, and 146, the vast majority of activating mutations occur in either the 12th or 61st codon (Downward, 1992; Brown et al., 1990). Both of these codons are in close proximity to β and γ phosphates of the bound GTP (Downward, 1992). These mutations cripple Ras’ ability to hydrolyze GTP, and therefore, Ras is constantly in the active state. In addition to GAP, the products of two tumor suppressor genes interact with Ras. NF-1, the product of the neurofibromatosis gene, and Rap, the product of the Krev-1 gene, appear to regulate Ras function (Downward, 1992). These alternative and redundant mechanisms of regulation may be responsible for the myriad of Ras responsive pathways.

Ras Signal Transduction: Looking Towards the Nucleus

By 1982, ras was identified as a central oncogene in both animal and human tumors. Its homology and intrinsic GTPase activity hinted at a signal transduction role. While ras has been shown to regulate the adenylate cyclase pathway in yeast, until very recently, the biological function of ras in mammalian cells was poorly understood (Barbacid, 1987). The identification of oncogenes acting at various subcellular locations suggested the possibility of an integrated pathway of action. For years, the products of extracellular, transmembrane, cytoplasmic and nuclear oncogenes have been known. However, while
individual oncogenes seemed to act as signposts directing signals to the nucleus, significant gaps in the proposed pathways and mechanisms existed. Several of these breaches appear to be overcome as recent discoveries may connect Ras signal transduction pathways to the nucleus.

For many years it has been known that treatment with extracellular growth factors stimulates an increase in the active form of Ras (Satoh et al., 1990; Gibbs et al., 1990). However, no direct associations between the growth factor receptors and Ras could be identified which would explain the stimulation of Ras. Recent results have identified two intermediary proteins (Marx, 1993). Briefly, these two proteins, GRB2 and mSos (mammalian homolog of the Drosophila son of sevenless gene also homologous to cdc25 in yeast), act to bridge the gap between growth factor receptors and Ras. Upon binding its cognate growth factor, growth factor receptors, themselves tyrosine kinases, autophosphorylate. GRB2 and mSos are cytoplasmic dimers, and are stimulated to bind phosphorylated growth factor receptors. The mSos protein then contacts Ras and activates it by stimulating the exchange of GDP for GTP. These results have helped clarify the transmission of a growth factor signal from the extracellular environment to the inner membrane.

New results tie Ras activation in with the stimulation of MAP kinases (Moodie et al., 1993; Van Aelst, 1993). MAP, or mitogen activated protein, kinases are known to act in cascades whereby one activates another. However, it was not known how the initial kinase is activated. Several novel papers suggest that another oncogene Raf-1, itself a
protein kinase, interacts with Ras, and then activates the first of the MAP kinases (Moodie et al., 1993; Van Aelst, 1993). While as yet there is no proof of a direct interaction, the proposed Raf-1 connection would theoretically result in the transmission of a Ras mediated signal to the nucleus. Once activated, the MAP kinase cascade phosphorylates and stimulates the activity of two other known oncogenes, Ets and Jun. These oncogenes, which are transcription factors, in turn elevate transcription of a subset of genes. These discoveries have made the road to the nucleus a little more clear.

**ras Activation in Animal and Human Tumors**

Clinical observations and established experimental animal systems support a multi-step model of carcinogenesis (Boutwell, 1974; Knudson, 1971). Mutational activation of the c-Ha-ras gene has been implicated in the development of both human and rodent epidermal tumors (Barbacid, 1987; Bos, 1989). In the two-stage model of mouse carcinogenesis, not only is the frequency of c-Ha-ras mutation high (Brown et al., 1990), but it is proposed to be an initiation event (Zarbl et al., 1985; Balmain et al., 1984; Roop et al., 1986). In contrast, c-Ha-ras mutations are a relatively infrequent event in human epidermal carcinogenesis. Brown et al. reported that 100% of 7,12-dimethylbenz[a]anthracene (DMBA)-initiated mouse papillomas possess codon 61 mutations (Brown et al., 1990), and yet only 10% of these papillomas will progress to malignant carcinomas (Hennings et al., 1986). Several lines of evidence indicate that the
frequency of \textit{ras} mutations is lower in malignant carcinomas than in benign papillomas (Brown \textit{et al.}, 1990; Corominas \textit{et al.}, 1989). Since carcinomas are thought to arise from pre-existing papillomas, these observations could indicate that \textit{c-Ha-ras} mutations are lost during malignant progression, or that carcinomas arise at a higher frequency from papillomas which do not contain a \textit{c-Ha-ras} mutation. The first possibility contrasts with most models of carcinogenesis whereby progression is proposed to occur through the clonal selection and retention of genetic events required for tumorigenesis. However, in support of the second prediction, several studies report that \textit{ras} mutations correlate better with tumor growth or differentiation than with malignancy (Corominas \textit{et al.}, 1989; Boukamp \textit{et al.}, 1990). Furthermore, the absence of \textit{c-Ha-ras} mutations in the majority of human tumors of the epidermis and head and neck epithelia (Lieu \textit{et al.}, 1991; Pierceall \textit{et al.}, 1991; Schroeff \textit{et al.}, 1990) suggests that in contrast to mouse skin, human skin carcinogenesis proceeds predominately through a \textit{ras} independent pathway. The predominance of \textit{c-Ha-ras} mutations in mouse papillomas may be a result of the acute exposure to a potent carcinogen. While exposure to environmental carcinogens is an established risk factor in human carcinogenesis, most human epithelia cancers may be the result of chronic exposure to weak carcinogens.

\textbf{Results}

The morphology of initiated and tumorigenic epidermal cells under LC conditions
was indistinguishable from that of growing parental cells (Figure 1 A-C compared to D), with the exception that 03, a poorly differentiated squamous cell carcinoma line, tended to display polar extensions (Figure 1C). Characteristically (Kulesz-Martin et al., 1985; Kulesz-Martin et al., 1988; Yuspa et al., 1981), tumor cells continued to maintain a proliferative subpopulation upon exposure to medium containing 1.4 mM Ca^{2+} (Figure 1 E-G) while parental cells underwent terminal differentiation (Figure 1 H). Since epithelial cells stratify and keratinizing tumor cells grow poorly in soft agar, there are no morphological correlates of malignancy in vitro for epithelial cells as there are for fibroblast cell models. Therefore, histopathology of in vivo tumors was utilized to assign stages of malignancy.

As shown in Figure 2 I-K, cell line 09 (I) produced well-differentiated benign papillomas, 05 (J) moderately-differentiated squamous carcinomas and 03 poorly-differentiated carcinomas at skin graft sites (K) and in metastases to the lung (L).

DMBA has been previously shown to consistently induce c-Ha-ras activation in both papillomas and carcinomas of the epidermis which is detectable as transforming activity in the NIH3T3 focus formation assay (Balmain and Pragnell, 1983; Balmain et al., 1984). To test whether the tumor cells of our keratinocyte model had similar focus forming activity, we examined the high molecular weight genomic DNAs isolated from all the cell lines. No highly refractile, disorganized foci typical of dominant Ha-ras transforming activity were present in cells transfected with epidermal cellular DNA in comparison to the carB positive controls (Figure 3). The carB cell line was derived from
Cells were cultured under LC conditions conducive to normal keratinocyte proliferation (A-D) or under HC conditions which permitted tumor cell growth (E-G) but induced terminal differentiation of normal cells (H). Cell lines are: A, benign papilloma 09; B, well differentiated squamous cell carcinoma 05; C, anaplastic squamous cell carcinoma 03; and D, nontumorigenic epidermal keratinocytes 291. Cells in the second row are in the same order only grown under high calcium conditions.
Figure 2.

Micrographs of Tumor Cells.

Micrographs depict hematoxylin and eosin stained sections of tumors obtained by skin grafting (I, 09, J, 05, K and L 03). All tumors were derived from the skin except (L) which depicts a lung metastasis of 03. Bars 125 μM (A-H), 100 μM (I), 120 μM (J), and 60 μM (K,L).
Figure 3.

Morphology of NIH3T3 Cell Foci Induced by Genomic DNA Transfection.

Passage twelve NIH3T3 fibroblasts were used as the recipient of genomic DNA isolated from non-tumorigenic, benign, or malignant cells. Mock transfections containing no DNA and transfection of carB DNA, which contains an activated c-Ha-ras, were used as negative and positive controls respectively. Transfected fibroblasts were maintained 21 days in DMEM containing 5% calf serum. Pictured are a portion of a highly refractile representative c-Ha-ras positive carB focus (A), a non-ras 05 focus (B), a non-ras 03 focus (C), and a background of confluent NIH3T3 cells (D). Bar ~ 75 μM
a DMBA/TPA induced squamous cell carcinoma and is known to contain a homozygous 61st codon A to T transversion (Buchmann et al., 1991). However, atypical foci were detected in all of the epidermal lines tested. Genomic DNA from each carcinoma cell line, 05 and 03, formed foci at 5 fold higher frequencies than 291 genomic DNA (Table 1). Genomic DNA for the benign papilloma forming cell line 09 generated foci at a 2.5 fold higher frequency than the non-tumorigenic line. These results correlate well with the *in vivo* tumorigenicity data.

A more direct search for point mutations in the frequently mutated codons 12, 13, and 61 of the c-Ha-ras proto-oncogene was carried out using the polymerase chain reaction technique. As shown in Figure 4, DNA from the carB positive control NIH3T3 foci contained the expected Xba I restriction fragment length polymorphism (RFLP) indicative of the transfer of c-Ha-ras gene with a 61st codon A to T transversion. In contrast, PCR-amplified DNAs from the non-tumorigenic 291, the papilloma 09, and the squamous cell carcinoma 05 and 03 cell lines were not sensitive to restriction by Xba I. Likewise, individual NIH3T3 cell foci from the carcinoma cell DNA transfections did not contain the Xba I RFLP (data not shown). Point mutations in the 12th, 61st (Figure 5) and 13th codons (data not shown) were ruled out by PCR amplification and dot blot hybridization of PCR products with sequence-specific oligonucleotide probes.

In the absence of coding mutations, c-Ha-ras activation can occur by either gene amplification or overexpression (Balmain *et al.*, 1984; Quintanilla *et al.*, 1986; Pelling *et al.*, 1988). Southern analysis indicated no amplification of the c-Ha-ras gene
Table 1.

DNA Transfections.

<table>
<thead>
<tr>
<th>Genomic DNA</th>
<th>Total Foci</th>
<th>Total Dishes</th>
<th>Total ug</th>
<th>Foci / ug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>3*</td>
<td>13</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>0</td>
<td>13</td>
<td>120</td>
<td>0.0</td>
</tr>
<tr>
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</tr>
<tr>
<td>291</td>
<td>12</td>
<td>21</td>
<td>142.5</td>
<td>0.08</td>
</tr>
<tr>
<td>09</td>
<td>11**</td>
<td>9</td>
<td>54</td>
<td>0.20</td>
</tr>
<tr>
<td>03</td>
<td>62</td>
<td>21</td>
<td>142.5</td>
<td>0.43</td>
</tr>
<tr>
<td>05</td>
<td>67</td>
<td>18</td>
<td>157.5</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Mock transfections containing no DNA and NIH3T3 DNA into NIH3T3 cells served as negative controls. Genomic DNA containing an activated c-Ha-ras oncogene, cHB, served as the positive control. 18 hours after transfection, plates were split and maintained in DMEM 5% calf serum for 28 days at which time foci were scored. Data is the cumulative result of three independent experiments except where noted.

* Total results from 2 experiments.

**Total results from 1 experiment
Figure 4.

PCR-RFLP Analysis for Putative A<sup>182</sup> to T Transversion in the 61st Codon of c-Ha-ras.

A 588 bp fragment containing the 61st codon of c-Ha-ras was amplified, subjected to Xba I digestion, resolved by 1.5% agarose gel electrophoresis, and stained with ethidium bromide. pBR322 DNA digested with Bst NI or Msp I was used as size standards. The small arrowheads mark the positions of the uncut, 588 bp fragment, and the Xba I fragments diagnostic of a 61st codon mutation, 414 bp and 174 bp.
Figure 5.

Differential Oligonucleotide Hybridization for the Detection of Putative Mutations in Codons 12 and 61 of c-Ha-ras.

PCR-amplified DNAs containing codons 12 or 61 were spotted onto nylon membranes and subjected to differential oligonucleotide hybridization as described in "Materials and Methods". LEFT: Hybridization with oligonucleotides containing substituted nucleotides in codon 61. Amplified DNAs were spotted in the following order: 1a, normal murine epidermis; 1b, line carB; 1c, strain 291; 1d, line 03; 1e, line 05; 1f, line 09; 2a, heterozygous mouse tumor with C$^{181}$ to A transversion; 2b, heterozygous mouse tumor with A$^{182}$ to T transversion; 2c, heterozygous mouse tumor with A$^{182}$ to G transition; 2d, heterozygous mouse tumor with A$^{183}$ to T transversion; and 2e, amplification without genomic DNA. Hybridizations were carried out with oligonucleotides specific for the wild-type sequence CAA of codon 61 or the mutated sequences AAA, CTA, CGA, and CAT. RIGHT: Hybridization with oligonucleotides containing substituted nucleotides in codon 12. Amplified DNAs 1a-1f were spotted in the same order as seen on the left side. 2a, heterozygous mouse tumor with G$^{33}$ to A transition; 2b, heterozygous mouse tumor with G$^{34}$ to A transition; 2c, heterozygous mouse tumor with G$^{34}$ to T transversion; and 2d, amplification without genomic DNA. Hybridizations were carried out with oligonucleotides specific for the wild-type sequence GGA of codon 12 or the mutated sequences AGA, GAA, GTA, and GCA.
Figure 5.

Differential Oligonucleotide Hybridization for the Detection of Putative Mutations in Codons 12 and 61 of c-Ha-ras.
in the non-tumorigenic, benign, or malignant carcinoma cell lines (data not shown). Furthermore, there was no difference between carcinomas and parental epidermal cells in the number of copies of c-Ha-ras alleles in foci derived from high molecular weight DNAs. Northern analysis indicated that the abundance of c-Ha-ras mRNA was similar among benign and malignant tumor cell lines and the 291 parental cells (Figure 6).

Discussion

Experimental animal systems and in vitro transformation assays designed to simulate human carcinogenesis have helped to provide both a functional and temporal framework for the operational model of multi-stage carcinogenesis. These systems have been used to identify chemically-induced gain-of-function mutations in cellular proto-oncogenes. The NIH3T3 transformation assay has been particularly efficient in detecting mutated c-Ha-ras, one of the most extensively studied oncogenes to date in both rodent and human tumors (Barbacid, 1987; Brown et al., 1990; Balmain and Brown, 1988; Balmain and Pragnell, 1983; Guerro and Pellicer, 1987). Analysis of foci formed in this assay frequently correlates dominant transforming activity with the transfer of a c-Ha-ras oncogene mutated at the 12th, 13th, or 61st codons.

The laboratories of Barbacid, Balmain, and Bowden have identified carcinogen-specific ras mutations in tumors derived by initiation/promotion protocols (Zarbl et al., 1985; Brown et al., 1990; Balmain and Pragnell, 1983; Bonham et al.,
291 normal parental and independent normal clone 271c cells were grown in conditioned LC medium to enrich for proliferating basal cells or in HC medium for 48 hours before harvesting to enrich for differentiating spinous cells. Tumor cell derivatives (09, 03, and 05) were grown in HC. The same blot was rehybridized with a $^{32}$P labeled 7S probe to serve as an internal control for RNA loading. The positions of the 28S and 18S rRNAs are indicated.
1989). In the mouse skin two-stage initiation/promotion model, DMBA can act either as a potent complete carcinogen or as an initiator inducing heterogeneous populations of benign papillomas with varying potential to progress to malignancy (Brown et al., 1990; Slaga, 1974). These data and the discovery that the v-Ha-ras oncogene can replace chemical initiation has led to the proposal that c-Ha-ras mutations may be coincident with initiation in mouse skin (Bailleul et al., 1990; Brown et al., 1986). However, in the absence of promoting events, initiated skin does not become neoplastic. The work presented in this chapter addresses the necessity and sufficiency of ras activation in carcinogenesis. The data is discussed in light of experimental animal and in vitro models. Tissue specific ras activation and its function in differentiation proliferation are considered. Finally, the role of selective pressures, derived from either the innate endogenous environment or exogenously applied as in tumor promotion, is discussed as a mechanism for the propagation of a given genotype.

**DMBA activates ras more frequently in animal than in cell culture models of carcinogenesis.**

Balmain *et al.* have reported that greater than 90% of DMBA-initiated benign mouse skin papillomas express a mutated c-Ha-ras oncogene (Balmain *et al.*, 1984). In fact, 100% of the benign tumors examined contained 61st codon mutations (Brown *et al.*, 1990). These data suggest that in this animal model system DMBA initiation is
essentially equivalent to the induction of a 61st codon mutation in c-Ha-ras. Extrapolating then, one can infer that in animals, DMBA-induced papilloma formation always proceeds through a c-Ha-ras activation pathway. However, this same laboratory has reported examples whereby DMBA initiation of cells in culture resulted in a transformed phenotype in the absence of ras activation (Quintanilla et al., 1991).

Our laboratory in collaboration with Kulesz-Martin's laboratory has recently confirmed that DMBA can initiate mouse keratinocytes in cell culture without c-Ha-ras activation (Schneider et al., 1993). The data from the NIH3T3 focus formation assay indicate that these cell lines possess dominant transforming activity (Table 1). However, oligonucleotide dot blot analysis of PCR amplification products indicated that c-Ha-ras was not mutated in the 12th, 13th, or 61st codons. While genomic DNA amplification of the c-Ha-ras oncogene or elevated c-Ha-ras steady-state mRNA levels are often indicative of activation of the c-Ha-ras gene (Balmain et al., 1984; Quintanilla et al., 1986; Pelling et al., 1987), the data presented here indicate that neither of these mechanisms can account for the dominant transforming activity detected in the NIH3T3 assay. Like the majority of human basal cell and squamous cell carcinomas (Lieu et al., 1991; Pierceall et al., 1991; Schroeff et al., 1990), these benign and malignant mouse cell lines appear to have originated independent of c-Ha-ras mutation. Similar reports have indicated that DMBA is more likely to activate c-Ha-ras in rat tracheal cells in animal models than in cell culture systems (Mass and Austin, 1989; Cosma et al., 1990). When viewed in this manner, it appears that DMBA initiates carcinogenesis via a ras activation
dependent pathway in animals and in a \textit{ras} activation independent pathway in cell culture. It seems unlikely that the frequency with which \textit{ras} is mutated by DMBA in cell culture could vary enough to account for these discrepancies. Thus if the assumption is made that DMBA mutates \textit{c-Ha-ras} genes with the same frequency in cell culture as in animal model systems, then some other mechanism must account for the differences in \textit{ras} activation frequencies observed.

In the context of my hypothesis, I propose that selective pressures innate to the model system examined are the driving force in determining whether initiated cells containing \textit{ras} mutations are propagated or are lost. In mouse keratinocyte cell culture, two particular conditions may directly affect whether cells with \textit{ras} mutations are selected for or selected against. One is the inclusion of growth factors or conditioned media. Epidermal growth factor (EGF) appears to have a significant role in this effect. Weissman \textit{et al.} have shown that \textit{ras} mutations correlate with EGF independence (Weissman \textit{et al.}, 1989), and Yuspa \textit{et al.} have reported that the inclusion of fibroblast conditioned medium as well as EGF greatly reduces the observed frequency of spontaneous \textit{ras} activation in culture (Greenhalgh \textit{et al.}, 1989). In this case, I suggest that mutated \textit{ras} genes provide a selective advantage by aiding in an escape from programmed senescence. The observation that EGF and fibroblast conditioned medium may suppress \textit{ras} activation frequencies in culture suggests that they share a common signal transduction pathway with \textit{ras}. If this were the case, then an activated \textit{ras} gene could supplant the necessity of exogenous growth factors. In fact, the observation that
keratinocytes enter a crisis period only after several weeks in culture supports the hypothesis that a critical growth factor becomes depleted in culture (Greenhalgh et al., 1989). Thus if the selective advantage that ras mutations provide in culture can be alleviated by EGF or conditioned media, then one would expect observed ras activation frequencies to decrease in their presence.

Extracellular calcium concentrations are a second condition of keratinocyte culture which may affect ras activation frequencies. The observation that keratinocyte cell clones resistant to high calcium (1.4mM)-induced terminal differentiation could be isolated from mouse skin epidermis after carcinogen treatment led to the suggestion that initiated cells possessed altered differentiation programs (Yuspa et al., 1981; Yuspa et al., 1985). This was further substantiated by the observation that both benign and malignant keratinocyte cell lines proliferate in the presence of 1.4 mM calcium while normal keratinocytes differentiate and die (Yuspa et al., 1981; Yuspa et al., 1985; Hennings et al., 1980). Several studies have also correlated ras activation with initiation (Zarbl et al., 1985; Roop et al., 1986; Bonham et al., 1989), and therefore, a suggested relationship between ras activation and a calcium resistant block to differentiation may appear highly attractive (Yuspa et al., 1985). However, important exceptions remain. Balmain reports that the use of the calcium switch technique in vitro after chemical initiation of keratinocyte cell culture rarely leads to ras activation (Quintanilla et al., 1991). Furthermore, Yuspa et al. have shown that while keratinocytes infected by sarcoma viruses carrying activated ras genes do exhibit a partial block to calcium induced
terminal differentiation, they are nonetheless growth arrested in high calcium (Yuspa \textit{et al.}, 1985). These data suggest that in cell culture, resistance to calcium induced differentiation and \textit{ras} activation could be separate genetic events. If this assumption is true, then these genetic changes should segregate independently. This appears to occur more frequently in cell culture than in rodent tumors, and may be a result of an exogenously applied selective pressure. The extracellular calcium concentrations in the local environment surrounding initiated cells is not exactly known, but it is unlikely that these initiated cells are challenged by a substantial increase in calcium concentrations following initiation. Therefore, if \textit{ras} activation is not absolutely required for resistance to calcium induced differentiation \textit{in vitro}, then this selection technique may actually select against cells with \textit{ras} mutations. A possible contradiction to this hypothesis comes from the Yuspa \textit{et al.} report that despite the presence of conditioned media, activated \textit{ras} genes were detected in primary cell cultures challenged by high calcium (Greenhalgh \textit{et al.}, 1989). This can perhaps be explained by their gradual step-wise selection technique which may have created a larger temporal window for co-accumulation of both genetic changes. In summary then, the utilization of the calcium switch technique in cell culture may act to eliminate cells containing \textit{ras} mutations. This selective pressure may not be present in animals, and therefore, the co-accumulation of both \textit{ras} activation and a block to differentiation is likely to occur.

\textit{Ras} activation is tissue-specific
When human and animal tumor types are grouped together, it is apparent that only one of the three ras genes is predominantly mutated in each type of tumor. In rodent model systems, c-Ha-ras is found activated in papillomas and carcinomas of the skin and carcinomas of the breast (Barbacid, 1987). In human tumors, activated Ki-ras is commonly associated with adenocarcinomas of the pancreas, lung, and colon while N-ras activation is associated with myeloid and lymphoid tumors (Bos, 1989). Since transcriptionally active genes are less likely to accumulate mutations than non-transcribed genes (Bohr and Wasserman, 1988), it is improbable that tissue-specific expression of ras genes determines which ras gene is most likely to become activated. It is perhaps more reasonable to presume that the individual ras gene products (Ha, N-, and Ki-) act in tissue specific signal transduction pathways. Therefore, if c-Ha-ras acts as the predominant modulator of ras mediated signal transduction in keratinocytes, then it would be expected that keratinocyte-derived tumors would express predominantly activated c-Ha-ras genes. The mouse fibroblast line NIH3T3 is very efficient at detecting the presence of activated c-Ha-ras oncogenes in DNA-mediated gene transfer experiments, and rarely are Ki-ras genes detected using this assay. This result may be related to the very large size of the Ki-ras gene compared to the c-Ha-ras gene. However, it is interesting to compare this observation with Yamasaki's report that while both c-Ha-ras and Ki-ras are activated by DMBA in vitro, 0/30 cloned transformed Balb/c foci contained activated c-Ha-ras oncogenes (Nakazawa et al., 1990). In fact, only cells containing Ki-ras mutations were recruited to eventually form foci (Nakazawa et al., 1991) Analogous to the keratinocyte
model system, perhaps Ki-ras participates in the major ras mediated signal transduction pathways of Balb/c fibroblasts, and thus Ki-ras mutations are predominant. This argues against the contention that susceptibility to mutation determines which ras gene is activated. What is perhaps more relevant is the primary role a given ras gene in the signal transduction pathways of a given cell type. This is best typified by the observation that despite the fact that only 20% of N-methyl-N-nitrosomethyurea initiated rat mammary cells contain activated c-Ha-ras genes, 83% of rat mammary tumors possess activated ras genes (Zarbl et al., 1985; Zhang et al., 1991). Thus it appears that the initiated cell with the most advantageous genetic changes is selected and recruited in a tissue specific manner.

**Does ras activation mediate both growth and differentiation?**

To determine why activated ras genes are more likely to be found in one model system than another, it would be most helpful if one could accurately predict the role of activated ras genes in the tumorigenic phenotype. In a number of animal model systems c-Ha-ras activation has been correlated with malignancy (Zarbl et al., 1985; Bonham et al., 1989; Balmain and Pragnell, 1983), and yet some tissue culture systems designed to model both human and animal carcinogenesis have failed to support this correlation. A lack of this correlation is exemplified by Fuesig's report that the *in vitro* introduction of a mutated c-Ha-ras oncogene into human keratinocytes correlated activated ras expression
with *in vivo* growth potential but not with malignancy (Boukamp *et al.*, 1990). In addition, Balmain's laboratory and others (Quintanilla *et al.*, 1991; Balmain *et al.*, 1988; Barrett *et al.*, 1986) have reported the existence of both keratinocyte and fibroblast cell lines which express activated ras oncogenes but are nontumorigenic. Indeed sometimes, ras activation may not correlate with either malignancy or growth potential, but rather with differentiation. Pellicer *et al.* have shown using either a human or rabbit keratoacanthoma tumor model, that activated c-Ha-ras oncogenes may have functional roles in terminal differentiation (Corominas *et al.*, 1989). These benign tumors consistently regress despite the continued expression of an activated c-Ha-ras oncogene (Corominas *et al.*, 1989). I propose that these examples are further evidence that the immediate cellular environment in addition to genetic changes determine the ultimate genotype and phenotype of an initiated cell. Thus the role of an activated ras oncogene, whether its involvement is to be in mediation of malignancy, growth potential, or differentiation, is dependent upon the selection pressures of its local surroundings. Ras acts as an effector participating in the transfer of a signal in a transduction pathway, but it is the immediate environment and other essential genetic changes that route and interpret the signal.

**Disappearing ras Mutations**

In both human and animal model systems, several studies have indicated a lower
frequency of ras mutations in malignant neoplasms than in their prerequisite benign precursors (Brown et al., 1990; Corominas et al., 1989). According to the multistage model of carcinogenesis, initiated cells pass through a benign stage before becoming malignant invasive tumors. By extrapolation then, genetic changes that define initiation and are required for carcinoma formation should be observable at every stage following initiation. Balmain has reported that 100% of DMBA initiated papillomas possess mutated ras oncogenes (Brown et al., 1990), and yet we know from other studies that less than 10% of these papillomas will progress to malignant carcinomas (Hennings et al., 1986). From this it can be inferred that ras activation is not sufficient for carcinoma formation. Indeed Balmain’s report (Brown et al., 1990), that 73% (11/13) of N-methyl-N'-nitroso-N-nitrosoguanidine (MNNG)-initiated papillomas contained activated c-Ha-ras oncogenes while only 15% (2/13) of the resulting MNNG-initiated squamous cell carcinomas possessed the same mutated gene. This observation could lead to speculation that in these tumors ras mutations are not even necessary for carcinoma formation. Balmain suggests that another sub-population of initiated cells, other than those containing ras mutations, are ultimately responsible for carcinoma formation (Brown et al., 1990). Further proof of sub-populations of initiated cells within papillomas comes from Yuspa’s report of excised and cultured papillomas (Harper et al., 1987). They report that when cell lines were established from six papillomas known to express a mutated c-Ha-ras oncogene, none of the established cell lines possessed activated c-Ha-ras oncogenes (Harper et al., 1987). This suggests that the original papilloma consisted of at least two
sub-populations: one with and one without an activated \textit{ras} oncogene. Thus the possibility remains that the observed selection pressures in cell culture may be similar to the selection pressures observed \textit{in vivo} as benign tumors progress to malignant carcinomas.

\textbf{Tumor promotion as a mechanism of selection.}

The most common and frequently applied exogenous selection pressure in models of carcinogenesis is tumor promotion. Tumor promotion is defined as the action and accumulation of changes, genetic or otherwise, that result in the progression of an initiated cell into the formation of a tumor. In the broadest sense, it is likely that endogenous or exogenous tumor promoters are the driving force behind the mechanism whereby genetic changes are propagated or lost. It has been proposed that \textit{in vivo} tumor promotion not only selects for initiated cells but at the same time against normal cells allowing for differential initiated cell outgrowth (Yuspa \textit{et al.}, 1982). In this same context, tumor promoters have been shown to be responsible for tissue specific carcinogenesis (Slaga \textit{et al.}, 1983). Thus the altered interaction and communication between normal and initiated cells undergoing \textit{in vivo} tumor promotion may be crucial in the selection process. While the majority of carcinogenesis models employ exogenously applied tumor promoters, it is likely in human carcinogenesis that the role endogenous tumor promoters is more important. However, \textit{in vitro} models of
carcinogenesis the interactions between tumor promoters, surrounding stroma, and initiated cells are often absent. These differences in cell-cell interactions may partially explain the discrepancies in respective \textit{ras} activation frequencies observed between animal and cell culture model systems. In fact, Balmain et al. and others have reported that when the \textit{in vivo} environment is partially recreated in cell culture, by the inclusion of fibroblast feeder layers, the observed frequency of \textit{ras} mutations more closely resembles \textit{in vivo} models (Quintanilla \textit{et al.}, 1991). Only by more closely investigating the role of tumor promotion in carcinogenesis and the continued elucidation of endogenous tumor promoters, can the mechanism of differential selection, propagation, and tissue specific recruitment of initiated cells be better understood.

Conclusions

The majority of human tumors are of epithelial origin. While most attempts to simulate human epithelia carcinogenesis \textit{in vitro} have met with difficulties, the use of rodent model systems has alleviated some of these difficulties and helped to elucidate the role of oncogene activation in carcinogenesis (Brown \textit{et al.}, 1990; Bonham \textit{et al.}, 1989; Guerro \textit{et al.}, 1987). However, human epithelial neoplasms may contain a different spectrum of activated oncogenes than mouse skin (Lieu \textit{et al.}, 1991; Pierceall \textit{et al.}, 1991; Schroeff \textit{et al.}, 1990). As discussed earlier, \textit{ras} activation is a frequent event in \textit{in vivo} mouse skin chemical carcinogenesis (Brown \textit{et al.}, 1990), and yet it is a relatively rare
event in human skin carcinogenesis. The development of malignantly transformed mouse keratinocyte cell lines independent of Ha-ras activation may help to identify important non-ras oncogenes. While activated ras has been proposed to complement other activated oncogenes in carcinogenesis (Weinberg et al., 1985; Greenhalgh et al., 1990), it is a very strong-acting oncogene in most functional transformation assays and may mask the identification of other weaker complementing oncogenes.

The acute experimental exposure of mouse skin to potent carcinogens as compared to chronic environmental exposure of human skin to indigenous carcinogens may account for some of the oncogene activation frequency differences. The majority of environmental human skin carcinogens are weak initiators, and the relatively long latency of tumor formation suggests that carcinogen exposure is chronic rather than acute. In vitro chemical initiation studies in diploid human cells has demonstrated a significantly greater resistance to tumorigenic transformation than rodent cells. In fact, in transformed human keratinocytes, Boukamp et al. have demonstrated that Ha-ras oncogene activation correlates better with growth potential than with malignancy (Boukamp et al., 1990). Another report demonstrated that ionizing radiation transformed immortalized human keratinocytes without activating the Ha-ras oncogene (Rhim et al., 1990). In addition, Schweizer's laboratory has found that only 1 out of 39 mouse squamous cell carcinomas induced by chronic UVb irradiation possessed an activated c-Ha-ras oncogene (data not shown). This further suggests that chronic exposure of mouse skin to carcinogens resembles human carcinogenesis. Furthermore, recent studies indicate that the ability to
detect c-Ha-ras mutations in mouse skin 30 days post initiation correlates with the potency of the carcinogen (Nelson et al., 1992). Quintanilla et al. have reported that ras activation is a much more frequent occurrence during in vivo mouse skin carcinogenesis than in in vitro studies (Quintanilla et al., 1991). These results suggest that ras activation may provide a greater selective advantage to initiated cells in mouse skin than in human skin.

While the present study suggests that DMBA induces genetic alterations independent of c-Ha-ras mutations, it does not rule out defects which may interfere with the c-Ha-ras signal transduction pathway. Other defects have been associated with malignancy in the cloned keratinocyte model, in particular, changes in gene expression of the p53 tumor suppressor gene and the endogenous retrovirus-like sequence VL30 (Han et al., 1990; Han et al., 1992). However, the events related to initiation in this model are still unknown. Since DMBA may affect a significantly larger spectrum of genes during chemical carcinogenesis than suspected to date, the cloned keratinocyte model may prove useful in elucidating further genetic alterations in keratinocyte transformation. The results obtained in these mouse cell lines suggest that the genetic events responsible for malignant transformation may be pertinent to the etiology of non-ras human skin tumors. Furthermore, since these cell lines lack c-Ha-ras mutations, future studies in this model system provide a focus for the identification of analogous functional genetic defects in human cancers of surface epithelia.

In proposing the hypothesis presented here, I have attempted to address the
inconsistencies in ras activation frequencies in animal and cell culture models of carcinogenesis. In the simplest terms, an initiated cell, regardless of whether it expresses an activated ras gene or not, must progress through a number of temporal stages and accumulate successive genetic changes in route to the expression of a fully malignant phenotype. I have examined four scenarios and endeavored to explain how different endogenous or exogenously applied selective pressures could account for the observations presented. However, in order for this hypothesis to have value beyond that of a theoretical exposition, it must be capable of being applied to specific circumstances. In this light, I suggest that a prediction of our hypothesis is that by altering target cells and applied cell culture selective pressures (i.e. the inclusion or exclusion of tumor promoters, growth factors, or chalones), a different spectrum of activated oncogenes would be observed even when the initiator remains the same. I propose that inconsistencies such as these can only be reconciled when cell culture carcinogenesis is modified to more accurately mimic the in vivo environment and its selective pressures. These modifications could include: the co-culture of target cells with the cells derived from the expected surrounding stroma, the limitation of exogenously applied selective pressures, and the development of a medium which contains physiological concentrations of growth factors and inhibitors. In addition, the placement of tumor cells into orthotopic environments in nude mice should allow for a more accurate representation of their true phenotype.

In conclusion, I propose that different selective pressures may account for the
differences in \textit{ras} activation frequencies observed in cell culture and animal model systems. These selective pressures include induced differentiation, endogenous and exogenous growth factors and stimuli, altered extra- and intracellular communication pathways, and programmed senescence. Two corollaries can be drawn from our hypothesis: the adaptability of the initiated cell and the permissibility of the environment selects the genotype of the resulting tumor cell, and the initial activation frequency of a given proto-oncogene is less important than the subsequent recruitment and contribution of that activated oncogene to a phenotype that is selectively advantageous. While mutations may or may not be a direct result of carcinogen treatment, the ultimate fate of the initiated cell is determined through a progression of tissue-specific recruitment and selection in the immediate milieu that the chemical carcinogen and the tumor promoter create.
The Model System and Hypotheses Addressed

All multistage models of carcinogenesis propose that multiple genetic lesions propel a single nontumorigenic cell through a benign state to clonal neoplasms. In the mouse skin model, chemical initiation and promotion result in benign papilloma formation. A low frequency of these tumors convert to malignancy with further promoter treatment. All of this can be visualized macroscopically. However, from a population standpoint, it is impossible to determine which papilloma will convert and which will not. Genetic evidence suggests that all papillomas are not equivalent, but so far, no genetic mechanism has been identified to account for malignant conversion. Furthermore, it is unclear at which stage in this model tumor suppressor genes are lost.

By utilizing a single cell lineage model, we have recreated in vitro multistage carcinogenesis in culture. A nontumorigenic epidermal cell was treated in vitro with 7,12-dimethybenz[a]anthracene and three variants were isolated: 1) a benign papilloma forming cell line, 09, 2) a highly undifferentiated locally invasive carcinoma cell line, 03, and 3) a less malignant well differentiated carcinoma cell line, 05. Like the majority of human epidermal tumors, these cell lines do not have a mutated c-Ha-ras oncogene. However, each of the variants possesses dominant transforming activity proportional to
each's *in vivo* malignancy. The 03 carcinoma line is highly invasive and the most tumorigenic *in vivo*, and it also is the only variant with a known p53 defect (Han and Kulesz-Martin, 1992). By creating somatic cell hybrids between the nontumorigenic cell line (291) and each of the variants (09, 03, and 05), the role and timing of tumor suppressor gene loss was assessed in multistage carcinogenesis.

**Introduction**

Tumor formation is microevolutionary process driven by multiple sequential genetic alterations. As discussed in earlier chapters, the oncogene theory has been proposed to account for gain of function mutations. The multistage model of carcinogenesis not only predicts that the genesis of cancer is a multistep process, but it hypothesizes that these steps occur in ordered, defined, and discrete stages (Boutwell, 1974). Dominant acting oncogenes arrived on the cancer scene 20 years ago, and provided the first molecular and genetic evidence for the multistage model (Huebner and Todaro, 1969).

In contrast to the smaller class of recently cloned and sequenced tumor suppressor genes, the oncogene family has firmly established a functional and temporal role in multistage carcinogenesis (Bishop, 1987; Bishop, 1991). In the mouse skin model of multistage carcinogenesis, activated *ras* oncogenes are the most frequently observed (Barbacid, 1987; Bos, 1989; Brown *et al.*, 1990). In fact, activation of Ha-ras is proposed to be sufficient to confer the initiated phenotype (Zarbl *et al.*, 1985; Balmain *et al.*, 1984;
Roop et al., 1986). Cotransfection studies have demonstrated that a number of dominant oncogenes can cooperate to transform primary epidermal cells or fibroblasts (Land et al., 1983; Weinberg, 1985; Weinberg, 1989). Thus oncogenes are involved in the inception and progression of malignancy. The amplification of N-myc or neu in tumors is a sign of poor prognosis. Clearly oncogene activation has a significant function in carcinogenesis. Despite this evidence, activated or altered oncogenes have been detected in a small percentage of human cancers, providing ample indications that the family of tumor suppressor genes are likely to be equally or more important.

Extensive familial cancer studies predicted the existence of the first cloned tumor suppressor gene. The RB gene is deleted or mutated in greater than (90%) of bilateral retinoblastomas (Heim and Mitelman, 1987). These insidious tumors strike mainly children from birth to about 6-7 years of age. The early onset of retinoblastoma infers a functional role for the RB gene in tumor formation. Like the Wilm's tumor suppressor gene, the RB gene is altered in relatively few non-retinoblastoma tumors (Weinberg, 1991). In contrast, another tumor suppressor gene, p53, appears to be altered or mutated in a wide spectrum of tumors and cell lines (Marx, 1993; Sager, 1992). The functional role of tumor suppressor genes at some stage of tumor formation is indisputable. Both RB and p53 gene replacements reversed frank malignancy (Weinberg, 1991). These studies, however, have provided little evidence as the temporal significance of tumor suppressor gene loss in multistage carcinogenesis. Patterns of loss of both DCC and FAP in human colorectal cancer implicate a role for these tumor suppressors in malignant
progression (Marx, 1993; Weinberg, 1991; Vogelstein and Kinzler, 1993). More recently, loss of two candidate tumor suppressor genes, NF-1 and NF-2, have been associated with the formation of neurofibromatosis (Marx, 1993; Kinzler and Vogelstein, 1993). In addition, a putative suppressor of metastasis, nm23, has also been cloned (Marx, 1993).

Despite these advances, the number of oncogenes, nearly 100, dwarfs the small collection of putative tumor suppressor genes (Marx, 1993). Today nearly ten putative tumor suppressor genes have been characterized, and the search appears to be far from saturated (Marx, 1993). Unlike oncogenes, however, the very nature of tumor suppressor genes makes their characterization difficult (Weinberg, 1991). In fact, the very existence of tumor suppressor genes is often postulated by their absence. This apparent paradox makes them difficult to study.

Methods for Detecting Tumor Suppressor Gene Loss

Five basic methods have been utilized for the identification of putative tumor suppressor genes (Stanbridge, 1990; Anderson and Stanbridge, 1993; Weinberg, 1991; Klein, 1987). These are somatic cell hybridization, microcell chromosome transfer, familial studies, loss of heterozygosity, and morphological reversion. Each method will be discussed briefly below.

Somatic cell hybrid studies have firmly established that nontumorigenic cells possess
and express tumor suppressor genes. The first somatic cell hybridization studies involved the fusion of mouse cell lines of high and low malignant potential (Harris et al., 1969; Klein, 1987; Stanbridge, 1990; Weinberg, 1991). Results from these studies were consistent with the theory that the malignant phenotype was dominant. Several years later Harris, Klein, and colleagues demonstrated that malignant and nontumorigenic mouse somatic cell hybrids were nontumorigenic (Harris et al., 1969). Based on these results, this group hypothesized that malignancy was in fact recessive. They reasoned that the difference between their experiment and the previous one was due to the generation of stable hybrids. That is, stable somatic cell hybrids are nontumorigenic and unstable hybrids revert to malignancy with the loss of chromosomes.

Subsequent somatic cell hybridization studies have identified individual chromosomes associated with the suppression of malignancy (Stanbridge, 1990; Anderson and Stanbridge, 1993; Weinberg, 1991; Klein, 1987). Microcell chromosome transfer has helped establish the existence and chromosomal location of putative tumor suppressor genes. A short representative list of tumors with the chromosomal location of associated putative tumor suppressor genes is found in Table 2.

Certain specific tumors appear to be hereditary. These cancers include retinoblastomas, Wilm’s tumors, familial adenomatosis polyposis, neurofibromatosis, and multiple endocrine neoplasias. In the case of retinoblastoma, 40% of the cases detected may be hereditary (Heim and Mitelman, 1987; Weinberg, 1991). In 1971, while studying these rare childhood tumors, Alfred Knudsen proposed that statistical analysis of tumor
Table 2.
Chromosomal Location of Common Tumor Suppressor Genes.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Chromosome Region</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder carcinoma</td>
<td>9q, 11p, 17p</td>
<td>Tsai et al., 1990.</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>1q, 11p, 13q, 17p</td>
<td>Ali et al., 1987; Chen et al., 1989; Devilee et al., 1989; Mackay et al., 1988.</td>
</tr>
<tr>
<td>Colorectal carcinoma</td>
<td>5q, 17p, 18q</td>
<td>Bodmer et al., 1987; Solomon et al., 1987; Vogelstein et al., 1988.</td>
</tr>
<tr>
<td>Melanoma</td>
<td>1,6</td>
<td>Dracopoli et al., 1989; Trent et al., 1990.</td>
</tr>
<tr>
<td>Retinoblastoma</td>
<td>13q14</td>
<td>Cavenee et al., 1983; Yunis et al., 1978.</td>
</tr>
<tr>
<td>Wilm’s tumor</td>
<td>11p13, 11p15</td>
<td>Koufos et al., 1984; Reeve et al., 1989.</td>
</tr>
</tbody>
</table>
incidence suggested a multi-hit hypothesis (Knudsen, 1971). More simply, he proposed two genomic lesions or "hits" were required for initiation of carcinogenesis. In short, he suggested that the retinoblastoma was the result of the loss of both copies of the retinoblastoma tumor suppressor gene. Like retinoblastoma, the study of other familial cancers, neurofibromatosis, Wilm's tumor, and familial adenomatosis polyposis, has led to the identification of other putative tumor suppressor genes.

In addition to familial studies, investigators have identified putative tumor suppressor genes through loss of heterozygosity on specific chromosomes (Stanbridge, 1990; Anderson and Stanbridge, 1993; Weinberg, 1991; Klein, 1987). This work has helped establish p53 and DCC as putative tumor suppressor genes. Breast, small cell lung, and colon cancer all display loss of heterozygosity on the short arm of chromosome 17. The common region lost in all of these tumors was found to contain the p53 gene. In another elegant study, Vogelstein and Fearon used a loss of heterozygosity on the long arm of chromosome 18 centered around 18q21-18qter to identify and clone a putative tumor suppressor gene (Weinberg, 1991). The gene termed DCC for "deleted in colon carcinoma" appears to be absent in most carcinomas but not adenomas of the colon.

The final method of tumor suppressor gene detection listed was morphological reversion. Noda and colleagues used this technique to clone the ras specific reversion gene Krev-1 (Noda et al., 1989). By transfecting cDNA libraries into transformed cells, they were able to identify potential tumor suppressor genes by distinguishing morphologically distinct revertant cells from transformed cells. Similar techniques have
also been used by other investigators to identify potential tumor suppressor genes in rat embryonic tissue (Schaefer et al., 1988).

**Mechanisms of Action for Tumor Suppressor Genes**

Tumor suppressor genes by definition are hypothesized to function by interfering with the tumorigenic process, but exactly how this is accomplished is still a matter of debate. It has been suggested that these genes may act as normal regulatory genes of proliferation and differentiation (Marx, 1993; Boyd and Barrett, 1990; Sager, 1992; Weinberg, 1991; Stanbridge, 1990; Klein, 1987). Bookstein and Huang have reported that reintroduction of the RB gene into a retinoblastoma cell line potently repressed growth in culture (Bookstein, 1990; Huang, 1988). Work by Stanbridge, Harris, and others indicates that induction of a normal differentiation program may be responsible for tumor suppression (Harris, 1985; Peehl and Stanbridge, 1982; Jaffe et al., 1992; Harris and Bramwell, 1987; Conway et al.; 1992). In any of the cases, it is not directly clear if tumor suppressor gene loss is an early, late, or random event in tumor formation.

Today many genes are being put forward as tumor suppressor genes (Marx, 1993). Frequently genes that are simply not expressed in specific tumors have been labeled tumor suppressor genes without being functionally tested (Marx, 1993; Sager et al., 1993). Nomenclature seems to have taken on a life of itself, and the true role and meaning of certain genes in carcinogenesis has become clouded as a result. In this introduction, I
have stressed the term putative tumor suppressor genes because in most cases confirmatory functional assays have not been completed. In cases where putative tumor suppressor genes have been re-introduced into deficient tumors, results have often been inconclusive, conflicting, and sometimes contradictory. In many cases, it is unclear what "tumor suppressor" actually means. Furthermore because of conflicting experimental evidence and theoretical models, the issue of dominant or recessive nature of malignancy has yet to be conclusively settled.

Despite these difficulties, it is clear that some phenomenon involving the suppression of the tumorigenic phenotype does occur (Marx, 1993; Boyd and Barrett, 1990; Sager, 1992; Weinberg, 1991; Stanbridge, 1990; Klein 1987). The mechanism of tumor suppression needs to be further investigated. Definitions and nomenclature in the oncogene and tumor suppressor fields need to be further clarified. Finally, further elucidation of the timing and role of tumor suppressor gene loss in multistage carcinogenesis will aid in the re-evaluation of the dominant or recessive nature of malignancy and further clarify the importance and necessity of oncogene activation in carcinogenesis.

Results

Selection and Evaluation of Somatic Cell Hybrids
The 291 cell line was derived from primary Balb/c epidermal cultures as previously described (Kulesz-Martin et al., 1988). Three tumorigenic variants obtained by DMBA initiation of 291 cultures in vitro produce either benign papillomas, 09, malignant well differentiated carcinomas, 05, or highly malignant locally metastatic undifferentiated carcinomas, 03. Each cell line was transfected with a plasmid conferring either G418 (pSV2neo) or hygromycin (pKCR3hygro) resistance. Isolated clonal populations were fused with polyethylene glycol. Resultant hybrids were selected in the presence of both hygromycin and G418 (Figure 7). Single clones were subcloned into 96 well plates and expanded. Five different combinations of parental cells were used to create somatic cell hybrids. In naming the hybrids, each parental cell was denoted by a single nonzero numeral such that 291 became 2 and 03 became 3 and so on. Thus in a hybrid between the nontumorigenic cell line 291 and the carcinoma forming 03, 291 x 03 was shortened to 23-n where n the number of the individual clone isolated. The following number of each somatic cell hybrids were cloned: four nontumorigenic x benign hybrids named 29, eleven nontumorigenic x carcinoma (six 23 and five 25), four benign x carcinoma 39, and four carcinoma x carcinoma 35. In addition all of the hybrids except for 25 were tested as pooled populations (e.g., 23-m, where m=pooled population). Figure 8 summarizes the somatic cell hybrids generated and the expected results.

In most cases, the hybrid cells tended to resemble its least tumorigenic parent. That is all hybrids which possessed a nontumorigenic parent closely resembled the orderly well defined cobblestone like appearance of normal epidermal cells in
Parental cells were transfected with drug resistance markers (pSV2neo and PKCR3hygro), co-cultured, and fused with polyethylene glycol \textit{in vitro}. In this illustration, nontumorigenic 291 cells are fused to malignant 03 cells. Dual drug selection allows for the outgrowth of only 291 \times 03 hybrids.
Somatic Cell Fusions and Their Predicted Phenotypes.

<table>
<thead>
<tr>
<th>Hybrids</th>
<th># of Individual Clones</th>
<th>Predicted Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>291 x 09</td>
<td>6</td>
<td>No Tumor Formation</td>
</tr>
<tr>
<td>291 x 03</td>
<td>7</td>
<td>Tumor Suppression?</td>
</tr>
<tr>
<td>291 x 05</td>
<td>5</td>
<td>Tumor Suppression?</td>
</tr>
<tr>
<td>09 x 03</td>
<td>5</td>
<td>Tumor Suppression?</td>
</tr>
<tr>
<td>03 x 05</td>
<td>6</td>
<td>???</td>
</tr>
</tbody>
</table>
culture (Figure 9).

All hybrids grew with relatively the same doubling time. Only the carcinoma x carcinoma hybrid, 35, had a phenotype unlike either of the parental cells. These hybrids were very bipolar and closely resemble cells from spindle cell carcinomas (Figure 9). In nearly all cases, the hybrids were initially considerably larger than either parental cell consistent with somatic cell hybrid formation (Figure 9). Some cultures displayed a large frequency of giant squamous cell indicative of either abnormally induced differentiation or extensive genetic damage (Figure 9). All of the hybrids are resistant to 400 μg/ml of G418 and 100 μg/ml of hygromycin which will kill all of the parental cells. Cytogenetic analysis of modal chromosome numbers was consistent with the observation that the clones derived were hybrids of parental cells (Table 3). In addition, northern analysis indicate individually cloned hybrids expressed both the neomycin and hygromycin resistance genes (data not shown).

**Suppression of Tumorigenicity**

The ability of the hybrid cell line to suppress tumorigenicity was tested using two different nude mouse assays. All hybrids were tested by subcutaneous injection into the flanks of nu/nu athymic Balb/c mice. Since it has been previously reported that papilloma cells will not grow in subcutaneous injection sites (Kulesz-Martin et al., 1988), nude mouse transplantation assays were also used to assess tumorigenicity. A cell line
In most cases, the hybrid cells tended to resemble its least tumorigenic parent. That is all hybrids which possessed a nontumorigenic parent closely resembled the orderly well defined cobblestone like appearance of normal epidermal cells in culture.
Table 3.
Modal Chromosome Numbers of Representative Parental and Somatic Cell Hybrids.

<table>
<thead>
<tr>
<th>Cell Line Tested</th>
<th>Sample</th>
<th>Modal Number</th>
<th>Range</th>
<th># Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>291 Nontumorigenic</td>
<td>291G</td>
<td>72</td>
<td>67-75</td>
<td>25</td>
</tr>
<tr>
<td>09 Benign Papilloma</td>
<td>09un</td>
<td>68</td>
<td>62-72</td>
<td>25</td>
</tr>
<tr>
<td>05 Malignant Carcinoma</td>
<td>05K</td>
<td>63</td>
<td>61-125</td>
<td>26</td>
</tr>
<tr>
<td>03 Malignant Carcinoma</td>
<td>03K</td>
<td>63</td>
<td>56-172</td>
<td>25</td>
</tr>
<tr>
<td>291 x 09</td>
<td>29-m</td>
<td>93</td>
<td>82-97</td>
<td>25</td>
</tr>
<tr>
<td>291 x 05</td>
<td>25-3</td>
<td>125*</td>
<td>60-143</td>
<td>25</td>
</tr>
<tr>
<td>291 x 03</td>
<td>23-5</td>
<td>93</td>
<td>71-100</td>
<td>25</td>
</tr>
<tr>
<td>03 x 05</td>
<td>35-m</td>
<td>112-116</td>
<td>84-120</td>
<td>23</td>
</tr>
<tr>
<td>09 x 03</td>
<td>39-3</td>
<td>122*</td>
<td>99-143</td>
<td>25</td>
</tr>
</tbody>
</table>

*Represents the mean chromosome number.
was judged to be tumorigenic if a nodule greater than 3 mm at its largest diameter was consistently seen. In 100% (12/12) of injection sites, the parental 03-hygro cell line was rapidly and aggressively tumorigenic, and 92% (11/12) of the sites of parental 05-hygro were tumorigenic (Table 4). In contrast, 0% of both the 291-neo (0/12) and 09-neo (0/14) were tumorigenic (Table 4).

All of the hybrids suppressed tumor formation as detected by sub-cutaneous injection (Table 4). Table 4 lists the number of tumors per site injected, the percentage of positives, and the mean latency of tumor formation. Most strikingly, none (0/10) of 25 (nontumorigenic x 05 carcinoma) hybrids were tumorigenic. Also none (0/12) of the 29 hybrids were tumorigenic. This was expected since neither parental cell line was tumorigenic phenotype after subcutaneous injection. Nearly half (6/14) of the 23 (nontumorigenic x 03 carcinoma) were suppressed. Interestingly, the benign papilloma cell line 09 was less effective at suppressing tumor formation than the well differentiated carcinoma cell line 05.

Because the papilloma-forming cell line, 09, will not grow sub-cutaneously, suppression of papilloma formation can only be detected in a transplantation assay. As represented in Table 5, each of the carcinoma controls, 03 and 05, formed tumors in 75% (3/4) of the sites tested. Consistent with previous reports, the papilloma-forming cell line, 09, also formed tumors in 75% of the injection sites (3/4). As in the sub-cutaneous assay, the 291 cell line remained completely nontumorigenic (0/4). Indicative of complete suppression of papilloma formation, none (0/22) of the transplanted 29 hybrids formed
Table 4.
Nude Mouse Sub-cutaneous Tumorigenicity Studies.

<table>
<thead>
<tr>
<th>Line</th>
<th># Clones</th>
<th>Total Sites</th>
<th>Total Positives</th>
<th>% Positive</th>
<th>Day 50% Positive*</th>
</tr>
</thead>
<tbody>
<tr>
<td>291</td>
<td>2</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>&gt;&gt;180</td>
</tr>
<tr>
<td>09</td>
<td>2</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>&gt;&gt;110</td>
</tr>
<tr>
<td>03</td>
<td>2</td>
<td>12</td>
<td>12</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>05</td>
<td>2</td>
<td>12</td>
<td>11</td>
<td>92</td>
<td>45</td>
</tr>
<tr>
<td>29 Hybrid</td>
<td>6</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>&gt;&gt;130</td>
</tr>
<tr>
<td>25 Hybrid</td>
<td>5</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>&gt;&gt;130</td>
</tr>
<tr>
<td>23 Hybrid</td>
<td>7</td>
<td>14</td>
<td>8</td>
<td>57</td>
<td>82</td>
</tr>
<tr>
<td>35 Hybrid</td>
<td>6</td>
<td>12</td>
<td>7</td>
<td>58</td>
<td>64</td>
</tr>
<tr>
<td>39 Hybrid</td>
<td>5</td>
<td>10</td>
<td>7</td>
<td>70</td>
<td>29</td>
</tr>
</tbody>
</table>

*This represents the day on which 50% of the sites formed tumors.
tumors. This is the first reported suppression of a papilloma phenotype *in vivo*. To further confirm complete suppression of the 25 hybrids as detected by sub-cutaneous injection, these hybrids were also transplanted. Again complete suppression was observed. None (0/12) of the transplanted hybrids formed tumors. In contrast to the tumors that formed from sub-cutaneous injections, those that formed from transplantation closely resembled tumors generated by classical *in vivo* chemical initiation (Figure 10). Histology of these tumors was consistent with previous reports.

**Induction of Differentiation**

It has been previously reported that nontumorigenic hybrids between human tumor cells and keratinocytes maintain an epidermal differentiation program and are induced to differentiate *in vivo* (Harris, 1985; Peehl and Stanbridge, 1982; Jaffe *et al.*, 1992; Harris and Bramwell, 1987; Conway *et al.*, 1992). It is well established that increased extracellular calcium concentrations (0.5-1.4 mM) induces differentiation and concomitant differentiation markers of primary epidermal cells *in vitro* (Hennings *et al.*, 1980; Yuspa and Morgan, 1981; Yuspa *et al.*, 1987). Under high calcium conditions, all three tumorigenic variants, (09, 05, and 03), continue to actively proliferate. However, the nontumorigenic 291 cells are induced to terminally differentiate and die within several days (Figure 11). Consistent with the induction of terminal differentiation, only the nontumorigenic 291 cells express the differentiation specific marker keratin K1 in
response to elevated extracellular calcium levels (Figure 12). In contrast, none of the tumorigenic variants (09, 05, and 03) were induced to express K1 (Figure 12). Consistent with this finding, I found that K10 expression was also induced in a panel of representative hybrids (Figure 13). To further test the hypothesis that induction of differentiation is the cause of tumor suppression in epidermal hybrids, I maintained all of the hybrids in either low (0.04 mM), medium (0.5 mM) or high (1.4mM) extracellular calcium concentrations. Phenotypically, each hybrid resembled its nontumorigenic cell line 291 when grown under similar conditions. Significantly, as demonstrated by the induction of the keratin K1 expression (Figure 12) or keratin K10 (Figure 13), the hybrids regained the ability to terminally differentiate in vitro.

Because the somatic cell hybrids appeared to regain the ability to differentiate in vitro, I was interested in the differentiation state of the tumors that did form in vivo. As expected, 100% (12/12) of the anaplastic carcinoma forming 03 cell line formed very undifferentiated carcinomas (Figure 14). Some of these tumors were so undifferentiated that the cell of origin was in question. All of these tumors metastasized to local axillary lymph nodes, most showed significant invasion along nerve sheath fibers, and some of the tumors showed dramatic distant cutaneous metastases. In contrast, all (11/11) of the tumors formed from the parental 05 cell line were well differentiated and no metastases were identified (Figure 14). In keeping with this pattern, the tumors that did form from the benign x carcinoma (39) hybrids were moderate to well differentiated and only 20% of these tumors had local metastasis (Figure 14). The tumors from the nontumorigenic
x carcinoma (23) hybrids tended to be poorly differentiated but less metastatic than the parental 03 cell line (Figure 14). Hybrids between the two carcinoma cell lines demonstrated no clear pattern of differentiation state, however, like the 23 hybrids there was a smaller frequency of local metastasis. Pathologically nonsuppressed hybrids had a range of phenotypes from very benign to invasively malignant. Second site cysts were identified on several animals ears suggesting that these hybrids may have been suppressed to a partially benign state initially. In addition, some sites showed either hyperkeratosis or benign papilloma formation while others range from very well to very poorly differentiated carcinomas (Figure 15).

I hypothesized that two possible explanations could account for in vivo tumor suppression: 1) the somatic hybrids could fail to survive either because they terminally differentiated or through another mechanism, or 2) the somatic hybrids could continue to proliferate in situ with a normal phenotype. Because I knew that the hybrids possessed the neomycin resistance gene, I used the polymerase chain reaction technique to discriminate between these two hypotheses. In both parental 291-neo cells and tumors that formed from nonsuppressed hybrids I was able to detect the neomycin gene (Figure 16). However, I was unable to detect this gene in any (0/4) sites where tumor formation was suppressed (Figure 16). This confirms a nontumorigenic pathology report, and suggests that a failure to survive in situ possibly due to terminal differentiation was responsible for tumor suppression.

To further test this hypothesis, nontumorigenic 291 cells, transfected with the
Table 5.
Nude Mouse Transplantation Tumorigenicity Studies

<table>
<thead>
<tr>
<th>Line</th>
<th>#Clones</th>
<th>Total Sites</th>
<th>Total Positives</th>
<th>%Positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>291</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>09</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td>03</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td>05</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td>291 x 09</td>
<td>6</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>291 x 05</td>
<td>5</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 10.

Morphology of *In Vivo* Tumors.

Micrographs of tumors that formed from unsuppressed hybrids.

A. Normal endogenous skin.
Figure 10.

Morphology of *In Vivo* Tumors.

Micrographs of tumors that formed from unsuppressed hybrids.

B. A very well differentiated tumor showing signs of acanthosis.
Figure 10.

Morphology of *In Vivo* Tumors.

Micrograph of a tumor that formed from an unsuppressed hybrid.

C. A moderately differentiated tumor showing keratin pearls
Figure 10.

Morphology of *In Vivo* Tumors.

Micrograph of a tumor that formed from an unsuppressed hybrid.

D. An anaplastic tumor showing some keratin pearls.
Figure 10.

Morphology of *In Vivo* Tumors.

Micrograph of a tumor that formed from an unsuppressed hybrid.

E. An anaplastic tumor with local metastases in blood vessels.
Figure 11.

Calcium Induced Terminal Differentiation of the 291 Cell Line.

*In vitro* micrographs of cell lines.

A. Nontumorigenic 291 cells grown in low calcium (0.04 mM).
B. Benign papilloma-forming 09 cells grown in high calcium (1.4 mM).
C. Malignant carcinoma-forming 03 cells grown in high calcium (1.4 mM).
D. Nontumorigenic 291 cells grown in low calcium (0.04 mM).
E. Nontumorigenic 291 cells grown in high calcium for 48 hours (1.4 mM).
F. Nontumorigenic 291 cells grown in high calcium for 72 hours (1.4 mM).
Figure 12.

Calcium Induced Expression of the Keratin gene Kl.

Somatic cell hybrids and parental cells were switched from low (0.04 mM) extracellular calcium concentrations to high (1.4 mM) extracellular calcium concentrations for 48 hours, and then total RNA was extracted. The northern blot was probed with a random prime labeled non-coding region of the mouse keratin K1 gene. Somatic cell hybrids and clone numbers are indicated across the top of the blot.
Figure 13.
Calcium Induced Expression of the Keratin gene K10

Somatic cell hybrids and parental cells were switched from low (0.04 mM) extracellular calcium concentrations to high (1.4 mM) extracellular calcium concentrations for 48 hours, and then total RNA was extracted. The northern blot was probed with a random prime labeled non-coding region of the mouse keratin K10 gene. Somatic cell hybrids and clone numbers are indicated across the top of the blot.
Figure 14.

Correlation of the Degree of Tumor Differentiation with the Probability to Metastasize.
Figure 15.

Micrographs of *In Vivo* Tumors Resulting from Transplantation.

A. Transplantation of nontumorigenic 291 cells results in normal skin outgrowth.
B. Transplantation of benign 09 cells results in well differentiated papilloma outgrowth.
Figure 15.
Micrographs of In Vivo Tumors Resulting from Transplantation.

C. Transplantation of malignant 05 cells results in well differentiated carcinoma outgrowth.
Figure 15.

Micrographs of *In Vivo* Tumors Resulting from Transplantation.

D. Transplantation of malignant 03 results in anaplastic undifferentiated carcinoma outgrowth.
PCR amplification of DNAs extracted from unsuppressed tumors or suppressed injection sites resulted in the detection of the neomycin resistance gene only in unsuppressed tumors. This gene was not detected in suppressed sites (hybrids 29-1, 35-1, and 23-m) and untransfected (09). These data are consistent with the hypothesis that suppressed somatic cell hybrids differentiate, stratify, and slough off the epidermis of the animal.
neomycin resistance gene, were transplanted onto the backs of nude mice. Transplanted mice were sacrificed at one day, one week, and approximately every week thereafter. Genomic DNAs were extracted from these sites and subjected to PCR analysis. Figure 17 indicates that these transplanted nontumorigenic cells are detectable one day post transplantation, but by one week no cells remain. These data are consistent with the hypotheses that these cells are induced to terminally differentiate in vivo, stratify, and are sloughed off the epidermis of the animal by one week.

Alternatively, tumor suppression could also theoretically occur through specific loss of dominant transforming activity. While such a loss is highly unlikely and selectively disadvantageous, it is impossible to rule out. Figure 18 illustrates the growth rate of nonsuppressed somatic cell hybrids. Tumors latencies were normalized such that all tumors were measured from the day of initial formation to the day of sacrifice. These data indicate that regardless of initial latency, once a tumor formed from a somatic cell hybrid, it grew at the same rate as parental tumor cells. This suggests that dominant transforming activity is not lost in suppressed hybrids, but masked by dominant tumor suppressor activity.

Discussion

Induction of Differentiation as a Mechanism of Tumor Suppression
PCR amplification of DNAs extracted from transplanted nontumorigenic cells transfected with the neomycin resistance gene resulted in the detection of the neomycin resistance gene only in the positive controls and cells transplanted for one day. This gene was not detected in transplantation sites harvested seven days later. These data suggest that nontumorigenic cells are induced to differentiate in vivo by one week, stratify, and are sloughed off the epidermis of the animal. Positive controls are: pRxneo, neomycin resistance plasmid; NMT, nude mouse tumor containing the transfected neomycin gene; 291neo, the nontumorigenic 291 cells that were used in the transplantation assay. Genomic DNAs isolated from two independent samples of mouse whole skins and the mock reaction containing no DNA were negative controls.
Figure 18.

In Vivo Tumor Growth Rates.

TUMOR GROWTH RATE

Tumors were measured from day of onset using calipers. In all cases, the largest diameter was recorded. Tumors latencies were normalized such that all tumors were measured from the day of initial formation to the day of sacrifice. These data indicate that once tumors formed, they all grew at essentially the same rate.
The somatic cell hybrids studied here revealed the presence of multiple tumor suppressor genes in a single cell lineage model of multistage carcinogenesis. In addition, these analyses have helped to place a functional and temporal significance on tumor suppressor gene loss in tumors of the epithelia. Peehl and Stanbridge demonstrated that when HeLa cervical carcinoma cells were fused to normal human keratinocytes, the loss of tumorigenic potential was associated with induction of differentiation \textit{in vivo} (Peehl and Stanbridge, 1982). Several reports have indicated that in tumor x fibroblast hybrids, tumor suppression is concomitant with fibroblastic differentiation (Harris, 1985; Harris and Bramwell, 1987). In these somatic cell hybrids, the nontumorigenic cell's differentiation program appears to have a dominant role. Hybrids differentiate just as though they were normal fibroblasts or keratinocytes. While earlier somatic cell fusion studies have been suggestive of an initial role for tumor suppressor gene loss in carcinogenesis, the studies presented here suggest for the first time that in epithelia cells a suppressive activity is lost in the earliest stages of cancer. My data showing tumorigenic suppression of carcinoma cells by nontumorigenic keratinocytes supports previous findings. I believe that this report details the first account of tumorigenic suppression by a benign cell line. While not as effective at suppressing tumor formation as the nontumorigenic cell, the number of tumors forming in benign x carcinoma hybrids is considerably less than the parental carcinoma cell line itself. Furthermore, the observation that nontumorigenic keratinocytes can suppress the formation of benign papillomas corroborates the hypothesis that tumor suppressor gene loss is a contributory
...genetic event in the formation of benign lesions in carcinogenesis.

The most difficult aspect in identifying tumor suppressor activity is the lack of a satisfactory *in vitro* assay for isolating single suppressed cells amid a vast population of rapidly proliferating tumorigenic cells. Morphological, biochemical, and physical characteristics have been used to identify suppressed clones (Marx, 1993; Boyd and Barrett, 1990; Sager, 1992; Weinberg, 1991; Stanbridge, 1990; Klein 1987). Despite the fact that suppressed hybrids differentiate *in vivo*, the majority of somatic cell hybrids possess a transformed phenotype *in vitro* (Stanbridge, 1990). Like the report of Harris and Bramwell (Harris and Bramwell, 1987), however, I was able to identify some indications of differentiation *in vitro*. In the majority of hybrids tested, increased extracellular calcium concentrations induced a partially differentiated phenotype *in vitro*. Most importantly I was able to detect the expression of the keratin K1 in response to elevated extracellular calcium levels. Expression of this gene is indicative that at least a population of the hybrids has regained the ability to terminally differentiate. The inability of PCR to detect neomycin resistance genes at transplantation sites of nontumorigenic 291 cells at one week is consistent with the hypothesis that induced terminal differentiation prevents colonization.

The data presented here demonstrate that multiple genetic changes are required for each discrete stage of carcinogenesis. Substantial evidence links oncogene activation with multistage carcinogenesis (Bishop, 1987; Bishop, 1991; and others). Activation of c-Ha-ras is proposed to be sufficient for initiation in mouse skin (Brown et al., 1986; Bishop,
While activation, rearrangement, or amplification of oncogenes occurs in a great diversity of both rodent and human tumors, the inactivation of tumor suppressor genes may be a more frequent event. *In vivo* multistage colorectal carcinogenesis provides evidence for the loss of four or five potential tumor suppressor genes while fewer oncogene events are associated with these malignancies (Vogelstein and Kinzler, 1993). My data support the hypothesis that gene inactivation is intrinsically involved in the initiation of carcinogenesis. The ability of both benign and heterologous carcinomas to suppress tumorigenicity, suggest that induction of terminal differentiation programs may be the mechanism of tumor suppression *in vivo*. The majority of benign papillomas in the mouse skin system are non-invasive well differentiated lesions that rarely convert to frank malignancy despite the presence of both transforming activity and activated oncogenes. Taken together with my hybrid data, this suggests that a key differentiation pathway is still intact. In addition, the fact that a well differentiated carcinoma was also capable of suppressing an anaplastic carcinoma implies that multiple pathways of differentiation and malignancy. The possibility of multiple pathways of malignancy strengthens the role of natural selection in pathogenicity of tumor progression. Time, carcinogen insult, as well as anti-tumor therapy provide both the pressure and means for the continued expression of increasingly malignant phenotypes.

**A Question of Dominance**
The fact that nontumorigenic keratinocytes can suppress carcinoma formation despite the continued presence and expression of activated oncogenes has raised the old mendelian question of dominance. Early oncogene experiments indicated that two oncogenes were sufficient to convert a cultured fibroblasts to malignancy (Land et al., 1983). More recent in vivo and in vitro evidence hint that many more genetic events are required for the conversion to malignancy (Vogelstein and Kinzler, 1993; Marx, 1993; Renan, 1993; Barrett and Anderson, 1993). Clearly there are circumstances where oncogenes behave in a dominant manner (Bishop, 1987; Bishop, 1991), however increasing evidence like the somatic hybrid data presented here demonstrate a recessive phenotype for activated oncogenes. I propose that while oncogenes may play a critical role in tumor progression, their role remains one of a recessive nature. Inactivation of tumor suppressor genes provides both a temporal and functional window through which activated oncogenes operate. Selective pressures imposed by the immediate environment ultimately determine the convoluted pathway to malignancy. The data presented here and elsewhere provide evidence to suggest that in a cell with truly all of its "normal" tumor suppressive activities intact, oncogenes are recessive. By extrapolation, it is reasonable to hypothesize that in vitro oncogene transfection experiments select for cell predestined for malignancy because of a previous tumor suppressor gene inactivation. Multiple oncogenes can only dominantly transform cells who have previously sustained one or more defects in their normal differentiation or proliferation pathways.
Conclusions

The results obtained in this murine model of multistage human carcinogenesis imply that tumor suppressor gene loss occurs early in carcinogenesis and preempts oncogene activation. These data further indicate that a differentiation defect is most likely required for tumorigenic progression. The evidence that multiple tumor suppressor genes may act at discrete stages in epithelial carcinogenesis may aid in a greater understanding of carcinogenesis and lead to more successful treatment of cancer.
The Model System and Hypotheses Addressed

The long latency of human tumors and the difficulty of transforming human cell lines has necessitated the investigation of rodent models of carcinogenesis. As stated previously, the model utilized for this work was designed to simulate human epithelial carcinogenesis. The importance of such a model is underscored by the realization that 78% of new cancers and 73% of deaths in 1993 will be from cancers of the epithelia (Boring et al., 1993). This model utilizes nontumorigenic murine keratinocyte cell line, 291, as the progenitor of tumorigenic lines. Initiation in vitro with the chemical carcinogen 7,12-dimethylbenz[a]anthracene generated tumorigenic cell lines. Subsequently, clonal cell lines were derived representative of distinct stages in the multistage model of carcinogenesis. One cell line, 09, forms benign papillomas upon transplantation onto the backs of nude mice. Another cell line, 03, forms malignant highly invasive carcinomas upon either transplantation or subcutaneous injection. These tumors are similar to human squamous cell carcinomas of the head and neck.

Using this model system, I hypothesized that progression of a nontumorigenic keratinocyte cell line through a benign state to frank malignancy involved the loss of
tumor suppressor genes. Specifically I was interested in identifying genes whose 
expression was downregulated or abrogated in the malignant carcinomas as compared to 
the nontumorigenic keratinocytes. To address this hypothesis, I utilized both differential 
northern analysis and a modified subtractive hybridization technique. These studies 
yielded two results. First, the northern analysis revealed that the keratin family of 
intermediate filaments was differentially regulated in this model system. These results 
were consistent with the theory that carcinogenesis involves aberrant or disrupted 
differentiation programs. Second, the subtractive hybridization technique identified five 
transcripts, SUZ 1-4 and 6, that were expressed exclusively by only the nontumorigenic 
cell line. One of these clones, SUZ 4, was also not expressed in the eight other 
tumorigenic cell lines tested. This clone hybridized strongly with a smaller sized 
transcript in murine hearts and was considerably homologous to a non-coding region of 
lactate dehydrogenase. While this technique does not address the role of these putative 
genes in tumor suppression, their expression patterns imply significance in the 
tumorigenic process.

Introduction

This chapter examines two not mutually exclusive mechanisms of carcinogenesis both 
of which rely on differential gene expression. The first mechanism involves the role of 
aberrant differentiation programs in carcinogenesis, and second involves abnormal gene
regulation in the form of loss of potential tumor suppressor gene expression. Differential northern analysis revealed that the expression patterns of the keratin family of intermediate filaments was consistent with aberrant or disrupted differentiation programs in tumorigenic cells. The function and role of keratins specifically in epithelial differentiation and carcinogenesis are discussed. In a latter section, the five sequences identified by subtractive hybridization are examined. Their expression patterns and potential roles in carcinogenesis are considered.

Cancer as a Disease of Aberrant Differentiation

Cancer is frequently regarded as a disorder resulting from unbridled proliferation. The identification of several oncogenes is consistent with this theory. Genes coding for growth factors or growth factor receptors have been shown to oncogenic when mutated (Cross and Dexter, 1991; Bishop, 1991; Bishop, 1987; Harris, 19991; Lewin, 1991; and others). Furthermore, genes encoding GTP binding proteins, nuclear DNA binding proteins, or a preponderance of tyrosine kinases have also been identified as oncogenes (Cross and Dexter, 1991; Bishop, 1991; Bishop, 1987; Harris, 19991; Lewin, 1991; and others). All of these products are consistent with proliferation or signal transduction pathways. The case for unregulated proliferation as the cause of cancer is strong. While oncogenes have been identified in a variety of tumor types, their functional role in tumorigenesis is commonly tested by transduction into immortalized and abnormal
fibroblasts. The significance of the recipient cell in these studies is a topic to be discussed later, but for the moment it is important to consider the difference between these cells and epithelial cells. *In vivo*, the majority of tumors arise from cells of the lining epithelia such as the skin. Skin is a complex epithelial structure with an innate differentiation program that appears to override proliferation signals. Psoriasis, for instance, is an example of hyperproliferative epithelial cells (Yuspa *et al.*, 1987). This disease is not considered neoplastic, however, because an unaltered differentiation program directs these cells to terminally differentiate. Many other examples suggest that cancer is not solely a disease of hyperproliferation. Teratocarcinomas revert to a nontumorigenic state when induced to terminally differentiate (Ruddon, 1987). Neuroblastomas can regress completely *in vivo* (Ruddon, 1987). In addition, the long latency period of most tumors suggests that hyperproliferation alone is insufficient to cause cancer. Furthermore, high proliferation rates do not always correlate with malignancy. Certain malignancies have low proliferation rates while normal tissues (e.g., mucosal crypt cells of the colon) or benign tumors (e.g., skin papillomas) have high proliferation indices. Evidence like this indicates clearly that carcinogenesis involves alterations or disruptions in normal differentiation pathways as well as unregulated proliferation.

Cancer is a disease of abnormal differentiation. Differentiation is the term given to the sum of all processes that result in the structural or functional cellular specialization. Self-renewing cells arise from a "stem" cell pool and ultimately they differentiate into the
specialized cells of the tissue. Differentiated cells perform the day to day functions required for the organism to survive. In general, these cells have reached a proliferative dead-end. Under ordinary circumstances, these cells will never divide. They simply function, age, die, and are replaced by identically differentiated cells.

The differentiation state of epithelial cells and its role in carcinogenesis is a major subject of this work. While unregulated proliferation is perhaps the trait most frequently associated with malignancy, increasing evidence points to a central role for the regulation of differentiation in carcinogenesis.

**Epidermal Differentiation Models**

The epidermis is the most exterior portion of the skin. While it only constitutes about one-fortieth of the skin’s thickness, it provides an essential protective function. A schematic representation of the skin is provided in Figure 19. Briefly, stratified epidermis is composed of four layers. The basal layer lies directly above the basal laminae. Basal epidermal cells are the only cells capable of self-regeneration. As a basal cell begins to differentiate, it loses its proliferative capacity and stratifies to form the suprabasal layer. As epidermal cells continue to differentiate, the suprabasal layer stratifies to form the granular layers, and then finally forms the stratum corneum.

The epidermis serves many functions. It helps the body maintain fluid and temperature homeostasis as well as providing an impenetrable barrier to microorganisms.
Figure 19.

Epidermal Differentiation.

Stratum Corneum
Granular Layer
Spinous Layers
  K1 / K10
  Loricrin
Basal Layer
  K5 / K14
Basal laminae
Vasculature
By maintaining a state of constant regeneration, it helps resist physical and chemical insults. The majority of the structural stability of epidermis comes from the keratin family of intermediate filaments.

Keratins, the most highly studied molecular marker of keratinocyte differentiation are a gene family of more than 30 independent proteins (Fuchs, 1990; Watts, 1991; Coulombe, 1993; Steinert, 1993). On the basis of amino acid composition, keratins are grouped into either type I acidic keratins (pK_i 4.5-6.0, K10, K13, K14, K18, and K19) or type II basic keratins (pK_i 6.0-7.5, K1, K5, K6, and K8) (Fuchs, 1990; Watts, 1991; Coulombe, 1993; Steinert, 1993). A type I keratin is always expressed with its type II partner, and 10,000-20,000 protein heterodimers oligomerize to form 10 nm intermediate filaments.

In normal epidermis, the basal cells not only are involved in self renewal but also in the generation of progenitors committed to terminal differentiation. Furthermore, basal cells or cells of the hair follicle are thought to be the targets of chemical carcinogenesis in vivo. Basal cells express predominately K5 and its partner K14 (Fuchs, 1990; Watts, 1991; Coulombe, 1993; Steinert, 1993). The mitotically active outer sheath root cells of the hair follicle, also expresses K5 and K14, but unlike basal cells, these cells frequently express K6, K16, and K19 (Coulombe et al., 1989). Expression of the K6/K16 keratin pair, the hyperproliferative keratins, is often associated with proliferative potential, wound healing, or malignancy (Finch et al., 1991; Kopan et al., 1989). As keratinocytes stratify, migration vertically off the basement membrane correlates with a loss of mitotic activity
and commitment to terminal differentiation (Fuchs, 1990). These suprabasal cells cease K5/K14 expression and initiate expression of the K1/K10 keratin pair (Fuchs, 1990). As these spinous cells become further stratified, keratin filaments accumulate and aggregate; loricrin and filaggrin expression contribute to the formation of the cornified envelope. Keratin filaments make up 85% of the protein content of the completely differentiated cell of the stratum corneum (Fuchs, 1990).

In tumors of the epidermis, this spatially and temporally ordered program of differentiation is disrupted. The inappropriate expression of K13 in mouse papillomas has been correlated with their malignant potential (Conti et al., 1990; Sutter et al., 1991). Most carcinomas of the epidermis express the simple epithelial-specific keratins K8 and K18 (Guerra et al., 1992; Cheng et al., 1990). Furthermore while a subset of cells in carcinomas express the keratin K1/K10 pair, most carcinoma cell lines in vitro fail to express this pair (Yuspa et al., 1987).

Differential Gene Expression and Cancer

While blocks in differentiation programs have significant roles in carcinogenesis, it has long been acknowledged that cancer was also a disease of aberrant gene regulation. Many oncogenes can transform cells by being overexpressed or inappropriately expressed (Cross and Dexter, 1991; Bishop, 1991; Bishop, 1987; Harris, 19991; Lewin, 1991; and others). Myc, for instance, is upregulated in leukemias and lymphomas, and Ha-ras is
overexpressed by many benign and malignant tumors. Similarly, potential tumor suppressor genes have been identified and isolated due to their lack of expression in certain malignancies.

Differential hybridization of cDNA libraries has been a useful technique for the identification of differentially expressed genes. This technique has been used to successfully isolate six genes whose expression is upregulated in benign papillomas and malignant carcinomas as compared to normal epidermis. However, this technique is not sensitive enough to detect relatively rare transcripts (Schweinfest et al., 1990; Herfort et al., 1991). In contrast, subtractive hybridization has been utilized to successfully identify and clone transcripts that are in very low abundance (Briehl et al., 1990; Lee et al., 1991; Wieland et al., 1990; Owens et al., 1991; Duguid et al., 1988; Sive et al., 1988). In an attempt to isolate and clone putative tumor suppressor genes potentially responsible for the tumor suppressor activity detected in somatic cell hybrids, I used a subtractive hybridization technique. This technique was modified to alleviate some technical difficulties, and to aid in the ease of use.

This modified procedure represents a rapid subtractive hybridization technique for the identification and subsequent cloning of rare, differentially expressed genes in tissue or cells of limited quantity. PCR amplification of cDNA libraries generates both target and driver sequences abrogating the need for large amounts of input RNA. This method is adaptable to directional and non-directional cDNA libraries. Photobiotinylation and avidin resin subtraction eliminates difficult hydroxylapatite chromatography steps. Direct
amplification of cDNA insert from cDNA libraries eliminates inefficient primer/adaptor ligation steps. Finally, post-subtraction PCR allows for the rapid and sensitive analysis of sequences previously in too low abundance to clone.

Results

Differential Expression of Keratin Genes

Figure 20 demonstrates that while other studies have correlated K6 expression with hyper-proliferation or malignancy (Finch et al., 1991; Kopan et al., 1989), in this in vitro model K6 is predominantly expressed by the non-tumorigenic and benign cell lines. The induction of K6 by calcium supports recent reports that the K6/K16 keratin pair is actually expressed by differentiating cells and not cells hyperproliferating (data not shown). Calcium is known to induce TGF-β, and TGF-β induces K6 in culture (Fuchs, 1990). Therefore, it is tempting to suggest that this pathway is directly responsible for K6 expression in 293 cells. While K6 is expressed at varying levels in the carcinoma cell line 03 in culture, the higher levels of K6 expression in an 03 malignant carcinoma are consistent with previous findings (Finch et al., 1991; data not shown).

The differential expression of K18 in the 03 cell line (Figure 20) is consistent with numerous reports correlating K18 expression with malignancy (Guerra et al., 1992; Cheng et al., 1990). The discovery that both TPA and 5-azacytidine induced K6 in the 09
Two independent samples of each cell line were grown to subconfluence in the presence of LC (Low calcium 0.04 mM) containing media. These results show that the K6 keratin transcript is expressed predominately in the non-tumorigenic 291, and benign 09 cell lines. In contrast, K18 is expressed, under the same conditions, exclusively by the malignant 03 cell line.
and 03 cell lines (Figure 21) suggest possible mechanisms of action. TPA is a hyperproliferative agent, and 5-azacytidine causes hypomethylation of CpG tracts. K18 was also induced in the 09 cells (Figure 21) by both TPA and 5-azacytidine, but not in the 291 cell line. Fos and Jun have previously been shown to regulate K18 expression \textit{in vitro} (Oshima, 1990), and while the induction of Fos and Jun is sufficient to explain K18 expression in the 09 cells, the lack of K18 induction in 291 cells indicates that other events are necessary.

Keratin K19 is commonly expressed in the outer root sheath cells of the hair follicle. The expression of K19 by the nontumorigenic, 291, and benign, 09, cell lines is suggestive that their progenitor cell was derived from outer root sheath cells of the hair follicle (Figure 22). The ubiquitous expression of the simple keratin K8 is in contrast to published reports (Figure 22). Keratin K19 has no endogenous pair keratin, and the K8 expression in nontumorigenic, 291, and benign, 09, cells may be linked to the K19 expression.

The induction of the differentiation-specific keratins K1, K10, and K13 by high calcium \textit{in vitro} (Figure 23) is consistent with a number of previous reports (Yuspa \textit{et al.}, 1987; Sutter \textit{et al.}, 1991; Yuspa \textit{et al.}, 1989). The inability of the benign papilloma-forming cell line or malignant carcinoma-forming cell line, to express K1, K10, or K13 (Figure 23) in response to high extracellular calcium levels confirms that these cell lines possess defects in their terminal differentiation program. Because c-Ha-ras activation is so prevalent in benign and malignant tumors of mouse skin (Brown \textit{et al.}, 1990), a
Cell lines were serum starved for 20 hours and then placed in either 100 ng/ml TPA or 5 mM 5-Azacytidine for the time points indicated. Panel A indicates that K6 was induced in the 09 cell line by both 5-azacytidine and TPA. In each case, K6 was induced at the first time point and expression levels remained relatively high throughout the time course. Panel B indicates that again both 5-azacytidine and TPA induced K18 in the 09 cell line; however, expression levels were lower than K6 expression. In this experiment, K18 was induced by 5-azacytidine at 20 hours with a relative peak at 28 hours. TPA induction of K18 began and peaked at the 1 hour time point. Panel C indicates that while both TPA and 5-azacytidine induced K6 in the 03 cell line, TPA was a stronger inducing agent. Both TPA and 5-azacytidine induced K6 at the first time point.
The keratin K19 gene does not have a dimerization partner and often is expressed concomitantly with K8 as seen here in the 291 and 09 cell lines. While K8 expression is often correlated with malignancy, these results indicate that its expression in this model is better correlated with K19 expression, a keratin expressed by mitotically active outer root sheath cells of the hair follicle.
As described previously, cell lines were grown in given calcium concentrations for 28 hours prior to harvesting RNAs. These results indicate that only the non-tumorigenic 291 cell line retains the ability to express the differentiation specific keratins K1, K10, K13 and loricrin in response to elevated extracellular calcium concentrations. Consistent with the hypothesis that these genes serve as markers of terminal differentiation, 291 cells grown in HC for 3-4 weeks cease all proliferative activity and frequently slough off culture plates. The inability of the papilloma-forming 09 or malignant carcinoma forming 03 cell lines to express K1, K10, K13, or loricrin in response to high calcium is consistent with their having a defective differentiation pathway.
number of studies have correlated c-Ha-ras activation with the induction of K8, K13, or K18 expression or the inability to express K1 or K10 (Guerra et al., 1992; Cheng et al., 1990). Like the majority of human tumors of the epidermis, these cell lines lack a mutated c-Ha-ras gene suggesting that ras activation is not necessary for the induction or maintenance of this phenotype.

In vivo, malignant tumors often show a relative decrease in the expression of the K5/K14 keratin pair as compared to proliferating basal cells (Trask et al., 1990). While K5 expression levels are decreased from levels observed in 291 cells, K14 expression levels are extremely low and it may not be expressed at all (Figure 24). The transcriptional uncoupling of a keratin gene pair is extremely rare.

291 cells could be induced to express the differentiation-specific keratins K1, K10, and K13 under low extracellular calcium concentrations when co-cultured with fibroblasts (Figure 25). The 03 carcinoma forming cell line regained the ability to express these same keratins when co-cultured with non-tumorigenic fibroblasts in high calcium (Figure 23). This suggests that fibroblast/keratinocyte interactions are directly involved in the induction of differentiation. Currently it is being investigated whether soluble factors secreted from fibroblasts or direct physical interactions are responsible for these results.

A summary of keratin gene expression patterns for all keratins tested is given in Table 6. Preliminary 2-D protein gel analyses indicated that the northern analyses accurately reflect the actual protein levels (Figure 26).
This figure indicates that both 291 and 09 cells express relatively higher levels of the K5/K14 keratin pair than the carcinoma-forming 03 cell line. In the case of both 291 and 09, K5 and K14 are expressed in relatively equivalent levels; however, while the 03 line expresses low levels of K5, K14 expression is nearly undetectable.
Co-culture of Epidermal Cell Line with Either Tumorigenic (T) or Non-tumorigenic (N) NIH3T3 Fibroblasts.

Panel A and B indicate that the keratins K1 and K10 are induced in the 291 cell line by co-culture with either tumorigenic or non-tumorigenic fibroblasts. Furthermore, extracellular calcium concentrations appeared to have no effect on the level of induction. While the 09 cell line was not induced to express either keratin under these conditions, the 03 cell line expressed both K1 and K10 when co-cultured with non-tumorigenic fibroblasts in high extracellular concentrations.
A. 2D-gel analysis of non-tumorigenic 291 cells grown in 0.10 mM Calcium for 48 hours. These preliminary gels were run by Dr. Ray Nagle’s laboratory and processed by Colette Witkowski of Dr. Anne Cress’ laboratory. This gel indicates that the following keratins proteins were detected: K5, K6, K13, K14, K15, and K16. These results are in general agreement with the northern analyses.
B. 2D-gel analysis of benign 09 cells grown in 0.10 mM Calcium for 48 hours. These preliminary gels were run by Dr. Ray Nagle’s laboratory and processed by Colette Witkowski of Dr. Anne Cress’ laboratory. This gel indicates that the following keratins proteins were detected: K1, K2, K4, K5, K6, K13, K14, K15, and K16. These results are in general agreement with the northern analyses.
C. 2D-gel analysis of malignant 03 cells grown in 0.10 mM Calcium for 48 hours. Representative keratin proteins are marked. These preliminary gels were run by Dr. Ray Nagle's laboratory and processed by Colette Witkowski of Dr. Anne Cress' laboratory. These results are in general agreement with the northern analyses.
Table 6.
Keratin Gene Expression Patterns.

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All cells were grown *in vitro* on tissue culture plastic in 5% FBS and 7.5% CO₂. LC=low calcium (0.04 mM) and HC=high calcium (1.4 mM)
**Subtractive Hybridization**

Subtractive hybridization is a powerful technique for the enrichment and subsequent cloning of genes predominantly expressed by one cell of a given tissue type. Investigators report that because a 100-1000 fold enrichment can be achieved, differentially expressed mRNA can be isolated in as low abundance as 0.001% (Schweinfest et al., 1990; Herfort et al., 1991). However, despite technological advances (Briehl et al., 1990; Lee et al., 1991; Wieland et al., 1990; Owens et al., 1991; Duguid et al., 1988; Sive et al., 1988), subtractive hybridization often possesses several innate difficulties. In general, the technique is highly labor intensive, technically demanding, and requires a large input of tissues or cells. The strategy presented here eliminates or lessens each of these difficulties and can be adapted to other model systems where the use of previously described subtraction techniques would not be possible. The technique presented here utilizes PCR of cDNA libraries. If appropriate pre-made cDNA libraries are unobtainable, this procedure requires only enough poly A+ RNA to construct representative cDNA libraries (1-5μg), and thus allows for the analysis of cells or tissues in limited quantity. Furthermore, this strategy accommodates the use of either directional or non-directional cDNA libraries. This method is extremely versatile and flexible because either freshly constructed or previously generated lambda or plasmid cDNA
libraries can be used.

PCR is used to amplify cDNA inserts directly from small library aliquots. A vector-specific primer, almost all commercially available vectors have sequencing primers (e.g., T7, T3, SP6, etc.) flanking the multiple cloning sites (MCS), and an oligo dT primer are used to directly amplify cDNA inserts. When directional cDNA libraries are used, a vector-specific primer that binds the opposite strand as an oligo dT primer is used (Figure 27). For non-directional libraries, two PCR reactions are performed. One reaction contains an oligo dT primer and a vector-specific primer to the right of the MCS (e.g., T3); a second reaction contains an oligo dT primer and a vector-specific primer to the left of the MCS (e.g., T7) (Figure 27). Thus each individual PCR reaction amplifies cDNA inserts as though the library was originally constructed directionally (Figure 28). Following PCR, these individual amplification reactions are pooled because an oligo dT-cellulose strategy described below will separate the poly dA-containing strands from those containing poly dT. The two PCR reactions must initially remain separate, however, to prevent the preferential amplification of the small region between the vector-specific primers in vectors that do not have cDNA inserts. The use of PCR and cDNA libraries not only alleviates the need for large amounts of input poly A+ RNA, but also provides for a virtually inexhaustible reservoir of starting material. While other subtraction protocols have utilized cDNA libraries, direct PCR amplification of cDNA inserts eliminates the time consuming task of either isolating single-stranded phage DNA or in vitro transcription of cRNA.
Figure 27.

Subtractive Hybridization Flowchart.

1. Vector-specific primer
2. cDNA Inserts
3. Oligo dt
4. Vector-specific and Oligo dt-primed PCR. Label by incorporating dCTP*
5. Denature and run PCR product over an oligo dt cellulose column.
6. Solution hybridize to single-stranded photobiotinylated poly A+ sequences
7. Subtract common sequences with avidin-linked resin. Phenol extract supernatant.
8. Post-subtraction PCR amplifies subtracted cDNA inserts in previously too low abundance to clone.
9. Radiolabel subtracted probe
Figure 28.
Gel Electrophoresis of PCR Amplification Products.

Lanes on the left are: 1 and 2, amplified cDNA inserts from a 291 lambda ZapII library; 3, 4, and 5, amplified subtracted 291 cDNAs; 6, size standards. Lanes on the right are: 1, negative control for PCR amplification, the lambda ZapII vector without inserts; 2, cDNA inserts amplified from a 291 lambda ZapII library; 3, amplified subtracted 291 cDNAs; 4, size standards.
PCR amplification of cDNA inserts generates not only target but also driver sequences for subtractive hybridization (Figure 28). Amplified poly dT-containing or poly dA-containing strands are separated and selected with an oligo dT-cellulose selection technique. Following PCR, individual or pooled PCR reactions are extracted with chloroform to remove mineral oil, boiled, and applied to an oligo dT-cellulose column. The poly dT-containing strands flow through while the poly dA-containing strands are retained by the column. After washing the column, the poly dA-containing strands are eluted off. This strategy allows the separation and selection of individual single strands thus generating both potential target and driver sequences.

Single-stranded poly dA-containing sequences recovered from the oligo dT-cellulose column serve as driver sequences and replace the need for excess poly A+ RNA. Photobiotinylation of single-stranded poly dA-containing strands allows for the use of an avidin-resin to remove both hybridized and unhybridized driver sequences for subtraction experiments. This biotin/avidin-resin subtraction technique also allows for simple centrifugation to replace tedious and difficult hydroxylapatite chromatography. Our experiments indicate that 98-99% of self: self hybrids are removed by avidin subtraction. When the driver and target sequences are related but non-identical (i.e. a carcinoma cell line [driver] derived from a non-tumorigenic cell line [target]) up to 96% of common sequences can be subtracted.

A final significant improvement presented here is post-subtraction PCR to increase
the sensitivity of the subtraction. Post-subtraction PCR allows for the entire subtraction reaction to be scaled down (0.1-1.0 μg of target sequence is sufficient). Post-subtraction PCR amplification also alleviates problems originating from poor post-subtraction recovery yields (Figure 28). This allows for the subsequent cloning of extremely small quantities of recovered product previously in too low abundance to clone or the generation of radio-labeled subtracted probes. The strategy of using a vector-specific and oligo dT primer to originally amplify cDNA inserts circumvents the inefficient step of ligating primer/adaptors to recovered product prior to PCR amplification. These steps allow for the analysis of target and driver pools that are not only rare but extremely similar.

**Isolation of SUZ 1-4, 6 and Unsubtracted Clone 32 (SUZ 5)**

The technique described above was used to subtract common carcinoma, cell line 03, sequences from the nontumorigenic line, 291, in an attempt to identify transcripts expressed exclusively or predominately by the nontumorigenic cells. Initial screens of approximately 50,000 with a subtracted probe plaques revealed 11 potential clones. These plaques were cored from the lambda plates and resuspended in phage dilution buffer. PCR of each of the eleven supernatants revealed inserts ranging from 500bp to several kilobases (Figure 29). The Lambda ZapII plasmid excision and rescue assay was utilized to obtain plasmid DNA. DNAs from each of the 11 potentially positive clones were
Panel A. Positive plaques were identified by hybridization of a 291 lambda ZapII library with a subtracted probe. Positive plaques were cored and suspended in phage dilution buffer. These supernatants were amplified using T3 and T7 primers. The small band at the bottom of the lanes represents amplification of the multiple site alone. Bands above this represent cDNA inserts. Lane 4B was a plasmid with a known 1 kb insert; lane pSKII- is Bluescript without any inserts; the remaining lanes are size markers.
Panel B. The gel run in panel A was transferred and probed with radiolabeled total 291 RNA. This autoradiograph indicates that these amplified cDNA inserts are expressed by 291 cells.
transformed, and six subclones of each of the original eleven were picked. Sixty-five clones in all were analyzed for inserts. Approximately 45% of these clones contained inserts. Six were chosen for subsequent northern analysis. These six clones, named SUZ 1-6, were hybridized against blots containing nontumorigenic, 291, benign, 09, or carcinoma, 03. RNAs. Figure 30 shows the result of the hybridization of this against clones SUZ 1, 3, 4, 6, and unsubtracted clone #32 (clone #32 was originally SUZ 5, but its expression pattern indicated that it was unsubtracted). Two of these clones were examined further. Figure 31 indicates that SUZ 4 and SUZ 6 hybridized only to nontumorigenic RNAs and not to any of tumorigenic cell line RNAs. Clone SUZ 4 was hybridized to a blot containing RNAs from five other laboratory tumor cell lines (Figure 32). As can be seen, this clone hybridizes only to the nontumorigenic cell line RNAs (291 and NIH3T3). In addition, this clone hybridized strongly to a smaller transcript in murine hearts (Figure 32). All six of the SUZ clones were preliminarily sequenced. SUZ 1, 2, 3, 5 (unsubtracted clone #32), and 6 showed no significant homology to any sequences in GENBANK. SUZ 4, however, demonstrated significant homology to a non-coding region of lactate dehydrogenase (Figure 33).

Discussion

Keratins as Markers of Tumorigenicity
Figure 30.

Northern Analysis of Four Subtracted Clones

Four potentially differentially expressed clones (SUZ 1, 3, 4, and 6) were used to probe a northern of total RNAs. Each of these clones was expressed exclusively by only the non-tumorigenic line 291. An unsubtracted clone #32 and the 7s RNA probe were used as loading controls.
Two clones, SUZ 4 and SUZ 6, were used to probe northerns of total RNAs from multiple samples of cell line RNAs. Cell lines were either untransfected (un) or transfected with either plasmids for hygromycin or neomycin resistance (hyg or neo). Consistent with previous results, each of these clones was expressed exclusively by only the non-tumorigenic line 291. An unsubtracted clone #32 and the 7s RNA probe were used as loading controls.
Panel A represents hybridization pattern of clone SUZ 4 against total RNA from a variety of other cell lines. Lanes are: 291, nontumorigenic keratinocytes; 03, malignant carcinoma; A6, malignant fibrosarcoma; NIH3T3, nontumorigenic fibroblasts; 308, benign papilloma; PDVC57, malignant spindle cell carcinoma; PDV, malignant carcinoma; CarB, malignant spindle cell carcinoma. This clone was expressed exclusively by the nontumorigenic cell lines only.
Panel B represents the hybridization pattern of clone SUZ 4 against total RNA isolated from a variety of nude mouse tissues. SUZ 4 is expressed in the dermis and a smaller transcript is strongly expressed in murine hearts. Whole skin, epidermis, muscle, liver and kidney show potentially low levels of SUZ 4.
Figure 33.

Sequence Homology Comparison Between SUZ 4 and Lactate Dehydrogenase.

<table>
<thead>
<tr>
<th>LOCUS</th>
<th>MUSLDHA</th>
<th>12851 bp ds-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEFINITION</td>
<td>Mouse lactate dehydrogenase gene, complete cds.</td>
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<td>lactate dehydrogenase-A.</td>
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<tr>
<td>SOURCE</td>
<td>Mouse DNA, (library of M. Edgell).</td>
<td></td>
</tr>
<tr>
<td>ORGANISM</td>
<td>Mus musculus . . .</td>
<td></td>
</tr>
<tr>
<td>SCORES</td>
<td>Init1: 160 Initn: 255 Opt: 365 69.0% identity in 242 bp overlap</td>
<td></td>
</tr>
</tbody>
</table>

**Suz**
AGGGCAATGACCATAGCCACGGAT-AATAGC::A-GGATAGTCA

**Musldh**
GTGGTCCATTTAGGGAAACGGGATCCACAGACAGTCAGGGTACGGGC:CAGGGAAACCCC

**Suz**
CTGCTCTGGTTGTTGGAGACCCACATGAAGACTGAGCTCGATATC

**Musldh**
CTACTCCAGTTGTTGGAGGAGATCATGAAGACCAAGCTGCTCATC

**Suz**
-----CCAGACCCAGGCTG-GTATGCTATTTGGTTGGTGGTTCAGTC7C7GAGAGGCTCCA

**Musldh**
GGGCCTAGATCCAGCCTGTGCTTGCTCTTTGGTTGGT-GTTCAGT7C7AGGAGCCCCCA

**Suz**
AGGGTCCTGTTAGTTGA-CNNTATGGCCTTCCTATGGAATTCCTGTTCCCCTTTGGAGCC

**Musldh**
AGGTTCAGGTTAGTTGATCCTGTTGGTCTTCCAGTGAAGTCCCTG-7CCTCTTTGGATGC

**Suz**
TTCGATCCTT-TCCCCATATCTTCCATAAGAG

**Musldh**
CTCAGAGCTCTCTCAGAATCTCCTCAATAAGAGCTCCCTGATCCTCAGAACTCTAACA

**Suz**
TAAGAGTCCCTT7-TCCATCCAAATGTTGGGTTGAAATCGCATCCTCATAAAGAATG
Three general conclusions can be drawn from the differential northern analysis of the keratin genes: 1) keratin gene expression patterns are suitable as markers of the tumorigenic phenotype in vitro, 2) the expression patterns of keratin K1, K10, K13 and the envelope gene Loricrin are consistent with the hypothesis that the tumorigenic cell lines have a terminal differentiation block, and 3) co-culture experiments demonstrated that while in vitro culture methods simulate in vivo epidermal growth, factors and structures supplied by other cell types and tissues are required to completely emulate in vivo stratification and growth.

These studies indicate that in this model system, the keratin gene K6 is a reliable marker of the nontumorigenic or benign phenotype. In contrast, K18 is expressed exclusively by the malignant phenotype. These patterns could be useful in identifying the tumorigenic phenotype of an unknown murine epidermal cell. The ubiquitous expression of the simple keratin K8 is in contrast to published reports. The K8/K18 pair is normally expressed only in simple embryonic tissues and frequently in malignant carcinomas. The expression of these keratins genes in tumors has been correlated to the expression of an activated ras gene (Guerra et al., 1992; Cheng et al., 1990). However, as discussed earlier none of these tumorigenic cell lines possess activated ras alleles. This suggests that their is not an absolute linkage between ras activation and K8/K18 expression. In fact, the K8/K18 expression may be the result of more general conditions attributed to carcinomas, e.g., up-regulated local TGF-α expression. Keratin K19 is commonly expressed in the outer root sheath cells of the hair follicle (Coulombe and Fuchs, 1989).
It has long proposed that the hair follicle might be the source of the "stem cell" population and thereby, may be the targets of *in vivo* chemical carcinogens. Keratin K19 has no endogenous pair and is often found expressed with K8. This may explain the unusual expression pattern of K8 in the nontumorigenic and benign cell lines. The transcriptional uncoupling of the keratin pair K5/K14 is an interesting finding and could have significant implications in carcinogenesis. These keratins are intricately involved in the formation of hemidesmosomes, and disruption of the normal structure of these keratin filaments could affect the way tumor cells adhere to their substrates.

The finding that only the nontumorigenic cell line 291 was capable of expressing the keratin genes K1, K10, and K13 in addition to the envelope gene Loricrin, is consistent with the hypothesis that a block to normal terminal differentiation is required for carcinogenesis. The fact that benign cells also will not respond to terminal differentiation signals in culture implies that this block is an early occurrence in carcinogenesis. While these data do not localize the timing of this block, data presented here and elsewhere imply that it occurs at or before initiation. Thus as indicated in the previous chapter, any genetic, endogenous, or exogenous influences that induced a initiated, benign, or malignant cell to differentiate could be a potent anticarcinogenic agent.

The finding that co-culture of 291 and 03 cell lines with nontumorigenic fibroblasts induced expression of the keratin K1 and K10 was intriguing. This result suggested that co-culture could induce these cells to differentiate in culture under conditions in which they wouldn’t normally, e.g., under low calcium conditions for 291, and high calcium
conditions *in vitro* for O3. This result was further investigated by growing each of these cell lines on plates coated with different components of the extracellular matrix. These components included fibronectin, laminin, collagen I, and collagen IV, none of which induced expression of either keratin gene. Growth on matrigel also failed to induce either gene. In addition, growth in fibroblast conditioned media also did not induce differentiation. It is likely that induction of differentiation was due to a combination of secreted and structural factors that successfully mimicked the *in vivo* environment.

**The Suz Clones**

Subtractive hybridization identified five potential genes whose expression was abrogated during the progression to malignancy. Their expression pattern alone warrants further investigation into a potential tumor suppressor function. The fact that SUZ 4 was also not expressed in five additional tumorigenic cell lines makes it an especially interesting clone. While the preliminary sequence analysis failed to identify any homologous genes for all clones with the exception of SUZ 4, this result may be due to the sequencing of primarily non-coding regions. The homology between SUZ 4 and lactate dehydrogenase is significant particularly in the light that it hybridizes to a small transcript in normal murine hearts. However, this homology is to a non-coding region of lactate dehydrogenase and its punctate nature clearly indicates that SUZ 4 is not lactate dehydrogenase. While the expression patterns of these clones is intriguing and exciting,
it is important to point out that no functional data are known. It is impossible to
differentiate between artifacts and true finds at this stage. The preliminary evidence is
promising and each clone warrants further study.

Conclusions

In summary, the data presented here support the hypothesis that this in vitro model
accurately simulates in vivo carcinogenesis. My results concur with the use of specific
keratin expression patterns as molecular markers of progression or malignancy. Finally,
this model may provide insight into the normal or aberrant regulation of keratin gene
expression during carcinogenesis.

This chapter details modifications to previously successful subtractive hybridization
techniques. This strategy is versatile and flexible because virtually any cDNA library can
used as a starting point. In addition, this method requires considerably less input poly A+ RNA, does not utilize hydroxylapatite chromatography, and utilizes PCR increased
sensitivity. These modifications result in a less labor intensive, straight forward method
for the isolation of rare differentially expressed genes. In particular, this technique was
successful in the identification and isolation of five potential genes whose expression was
restricted to nontumorigenic cell lines. These clones may have a role in tumor
suppression or normal development of epidermal differentiation, and further study may
identify significant cancer related genes.
IX CONCLUSIONS

The test of a first-rate intelligence is the ability to hold two opposed ideas in mind at the same time and still retain the ability to function.

F. Scott Fitzgerald
From, In Search of Excellence

Genetic Alterations: The Role of Oncogenes

The argument implicating cancer as a genetic disease is compelling. Ever since it was first speculated, scientists have searched for cancer causing genes. Today many people believe that these "cancer causing genes" have been found. In the broadest sense, these genes have been lumped into two categories: oncogenes and tumor suppressor genes. In the research presented in this dissertation, I have examined the function, role, and importance of each class of these genes in the genesis of cancer.

It is the goal of all models of carcinogenesis to elucidate or clarify some mechanism or phenomenon associated with the cause of predisposition of cancer. The direct observation of cancer or cancer causing agents in humans has been difficult. In the first place, most human cancers have long latencies, making the direct observation of multiple generation nearly impossible. Secondly, human cells are recalcitrant towards in vitro transformation. Some of these difficulties have been circumvented through the generation and use of rodent models of carcinogenesis. These models have helped establish a
multistage theory of carcinogenesis, and one of the goals of this work was to address the applicability of the murine model presented here to human cancer.

In humans, the majority of neoplasms arise from the epithelial linings of the body presumably because of prolonged contact with physical, radiation, or chemical carcinogens. Utilization of mouse skin models of carcinogenesis have helped identify a multitude of carcinogens. The carcinogen utilized in these studies, 7,12-dimethylbenz[a]anthracene (DMBA), is particularly significant to human carcinogenesis (Tannock and Hill, 1987). Originally isolated from coal tar, DMBA and cyclic polyaromatic hydrocarbon compounds like it are potent carcinogens. Coal tar was once used in dandruff shampoos, and polyaromatic hydrocarbons are significant carcinogens found in all tobacco products.

In mice, DMBA can act as either an initiating or complete carcinogen generating either benign or malignant neoplasms (Brown et al., 1990). Nearly 100% of all murine tumors generated in vivo by DMBA treatment contain activated ras oncogenes (Brown et al., 1990; and others). This very same oncogene was the first identified in human tumors. The very high frequency of ras activation in DMBA initiated tumors has led to the theory that this is the initial casual event. I investigated this hypothesis in context of a murine in vitro model of carcinogenesis.

DMBA initiation of nontumorigenic murine keratinocytes (291) in vitro resulted in the generation of both benign (09) and malignant (03 and 05) cell lines. Resultant tumors were histologically similar to human squamous cell neoplasms of the head and neck, and indistinguishable from tumors generated in vivo by classical two-stage carcinogenesis.
These cell lines were shown to possess transferrable dominant transforming activity. NIH3T3 foci generated in this assay did not resemble foci known to contain activated Ha-ras oncogenes. To further clarify the supposition that these tumors arose independent of ras activation, northern, Southern, Xbal PCR RFLP, and PCR discriminative oligonucleotide dot blot analyses were conducted. Each of these studies confirmed the initial finding and led to the conclusion that in this model system DMBA carcinogenesis proceeded in a Ha-ras independent pathway.

Initially this result appeared to be directly in contrast to in vivo models of murine carcinogenesis. However, subsequent reports (Quintanilla et al., 1991) corroborate this finding. In addition, ras activation appears to have a diminished role in human skin carcinogenesis (Bos, 1989; Pierceall et al., 1991; and others). In the context of examining the driving force behind the perpetuation and selection of genetic changes responsible for carcinogenesis, I propose that inconsistencies in ras activation frequencies in different animal and cell culture models are a result of different local environmental selective pressures. Local selective pressures are likely to be result of a conglomeration of factors made up of the local environment, physical or chemical insults, exogenous or endogenous tumor promoters, and genetic predispositions. This led me to propose the theory that the adaptability of an initiated cell in its interaction with its immediate environment may ultimately determine the genotype of the resultant tumor. In the latter section, I consider the sufficiency and necessity of oncogene activation in carcinogenesis.
Genetic alterations: The Role of Tumor Suppressor Gene Inactivation

The somatic cell hybrid studies of Harris and Klein first suggested that carcinogenesis consisted of more than simply oncogene activation (Harris et al., 1969). In addition to examining the role of oncogene activation in the model system presented here, I also investigated the potential role of tumor suppressor gene loss in carcinogenesis. Somatic cell hybrid studies have implicated the loss of putative tumor suppressor genes in a multitude of tumors. Several studies have even demonstrated the existence of tumor suppressor genes in epithelial cell lines. However, to date somatic cell hybrids had only been generated between malignant and nontumorigenic cell lines; benign cell lines have never been tested. By utilizing a single cell lineage model of carcinogenesis, I have attempted to characterize the temporal importance of tumor suppressor gene loss. That is, while it has been theorized that carcinogenesis requires the loss of multiple tumor suppressor genes, the timing of tumor suppressor gene loss in the context of multistage carcinogenesis is still in doubt. Oncogene activation is proposed to be coincident with tumor initiation, but what is the result of tumor suppressor gene inactivation? Are they inactivated prior to initiation? Does the formation of benign papillomas involve inactivation of tumor suppressor genes? Are multiple tumor suppressor genes inactivated in the progression of a normal cell to malignancy? It was questions like these that I sought to answer.

Consistent with previous studies, I demonstrated that nontumorigenic epidermal cells
were capable of suppressing malignancy. Significantly, one of two malignant cell lines was completely suppressed as judged by both subcutaneous and transplantation assays. In addition, the number of local metastases was also suppressed in these hybrids. Interestingly, hybrids between the two malignant cell lines were also to some degree suppressed indicating multiple pathways of carcinogenesis.

By creating nontumorigenic x benign and benign x malignant somatic cell hybrids, I was able to demonstrate a loss of tumor suppressor activity in the generation of benign papillomas. Nontumorigenic x benign hybrids were completely suppressed in transplantation assays, and benign cells were also capable of suppressing malignancy. To my knowledge, this is the first indication of inactivation of tumor suppressor genes in benign papillomas.

Hybrids that were not suppressed tended to form tumors that were less metastatic and more differentiated than parental tumors. These data imply the possible existence of a putative suppressor of metastasis and suggest that induction of differentiation may be the mechanism of tumor suppression in vivo. Consistent with this hypothesis, hybrids appeared to differentiate in vitro, like the nontumorigenic cell line, when exposed to increased concentration of extracellular calcium. In addition, in concordance with the hypothesis that the mechanism of suppression was induction of differentiation, hybrids expressed the terminal differentiation markers, keratins K1 and K10, in response to differentiating conditions.

These results imply an important role for tumor suppressor genes in all stages of
carcinogenesis. By utilizing a modified subtractive hybridization technique, I attempted to isolate putative tumor suppressor genes responsible for the tumor suppressive activity described above. In addition, I analyzed the role of differentiation in both the genesis and suppression of cancer. The conclusions form these studies are considered below.

Abnormal Differentiation in Benign and Malignant Cell Lines

The long latencies of most cancers have helped lead to the proposal that disruption of normal differentiation is the rate limiting step and not unregulated proliferation. The data presented here are consistent with this hypothesis. The nontumorigenic keratinocyte cell line 291 was induced to differentiate in culture in the presence of elevated extracellular calcium concentrations. Morphologically, these cells became less refractile than dividing cells, and within 24 hours squame formation marked the onset of terminal differentiation. After 72, no mitotic figures could be seen, and cells began to slough off the culture plate in sheets. These same cells were induced to express keratin genes (K1, K10, and K13), and cornified envelope genes (loricrin and transglutaminase) consistent with the concept that they were terminally differentiating. In contrast, benign and malignant cell lines derived from this initial cell line were not induced to differentiate and continued rapidly proliferating under the same conditions. None of terminal differentiating markers were induced. These results are congruous with other published data and strongly support the hypothesis that benign and malignant cells possess genetic blocks making them incapable
of terminally differentiating.

Further keratin studies demonstrated differential expression and helped establish specific keratin genes as markers of a given tumorigenic stage. In concordance with other published results, the malignant keratinocyte cell line 03 exclusively expressed the simple keratin K18. K8, the partner of K18, was the only keratin gene examined which was ubiquitously expressed. Published reports have correlated expression of the K8/K18 pair with the expression of an activated ras oncogene. However, the data from these studies indicate that this linkage is not absolute.

In contrast to the malignant cell line 03, the nontumorigenic line 291 failed to express K18 and instead expressed exclusively the keratin K6, thus making these keratins suitable markers of tumorigenicity. Expression of K19 by the nontumorigenic 291 and benign 09 cell lines may reflect an outer root sheath hair follicle cell origin for these cell lines. It has long been speculated that such cells may represent a 'stem cell' population and may be the targets of in vivo chemical carcinogens. These data support this hypothesis and add further justification for use of keratin gene expression patterns in establishing tumorigenic phenotypes in vitro.

Subtractive Hybridization Reveals Differential Gene Expression

Somatic cell hybrid studies supported the hypothesis that multiple tumor suppressor activities appeared to be lost during tumor progression. However, these studies could only
detect potential tumor suppressor activity, but could not be used to identify individual genes. To address this, I developed a modified subtractive hybridization technique. With this technique, I have identified five transcripts that are exclusively expressed by the nontumorigenic cell line 291. One of these clones, SUZ4, was also not expressed by five other tumorigenic cell lines tested. However, this clone did hybridize strongly to a smaller sized transcript in normal murine hearts. Partial sequence analysis revealed 70% punctate homology to a non-coding region of lactate dehydrogenase. This analysis indicated that this transcript may be related to but was clearly not identical to lactate dehydrogenase. This expression pattern of this clone and the others is consistent with that of a potential tumor suppressor gene. However, further sequencing and activity studies in malignant cells will be required to establish its function.

**Paradigms and Perspectives**

In the spring of 1985 when my interest in cancer was building, and two years before my graduate career began, I first read a Science article entitled, "Activated Proto-onc Genes: Sufficient or Necessary for Cancer?" (Duesberg, 1985) At the time, I felt it posed significant questions. Today it seems that the "Sufficient or Necessary" is an overworked trite colloquialism. The intent appears to have been lost and only the phrase remains. I believe the concept deserves a closer examination. Sufficient? Surely, the research conducted since 1985 have answered this question. The only genes, oncogene or
otherwise, that have been independently able to transform cells are the viral oncogenes. However, as will be discussed below, it is unclear if even these genes are acting independently. Nonetheless, to date no other oncogene has been demonstrated to be sufficient to cause cancer. Indeed, the idea itself seems to be logically and evolutionarily unsound. Why would an organism maintain a gene for millions of years that by itself could kill its host? Upon examining the "Necessary" clause of the phrase, more questions arise. A necessary change implies a change that is required. If one considers the oncogene family as a whole (over 100 members as of 1993) and adds the tumor suppressor family for good measure, these abnormalities are still found in the minority of tumors. Certainly no one event is required for all tumors. Specific tumors, however, do exhibit compelling patterns. RB gene loss in retinoblastomas, myc activation in B-cell lymphomas, or as applies to the work here, Ha-ras activation in murine skin tumors. Perhaps a better question to ask then would be "Why the strong correlation between this gene and tumor formation?" In examine this question, I think it becomes necessary to reconsider certain experimental assays and definitions.

Shakespeare once asked what was in a name. Names are given to objects out of both convenience and necessity. Hopefully, it is not too naive to expect that a name aids in a greater understanding of the object. When the oncogene hypothesis coined the term "oncogene," the definition of the word was relatively clear. Today, however, the picture is considerably muddier. The once powerful viral oncogene p53 is now a tumor suppressor gene or is it an apoptosis gene. Myc may be in the same boat. The endogenous ras
oncogene has been postulated to be a tumor suppressor gene. Mutated actin has been labeled an oncogene. The tumor suppressor nomenclature is not without its own difficulties. nm23 is a nucleotide diphosphate kinase, a transcription factor, but does it really suppress metastasis? The experts are arguing. Retinoblastoma gene replacements don't always suppress tumor formation. Why not?

It is very easy to stand on a mountain top and cast stones at everyone who is climbing up. However, lately in science that does not seem to be a large problem. To quote Nature editor John Maddox, today's molecular biologists seem more intent on "the enumeration of the molecular components of cells . . . collecting the details of new genes, and of new nucleotide sequences to go with them; new protein and their amino acid sequences; and novel membrane protein molecules, channels or receptors as they may be," than upon the meaning of such discoveries (Maddox, 1993). In fact, he links this apparent lack of thought to today's reward system, "It is less defensible that the practitioners appear to think less deeply about the meaning of the present abundance of data than is the case in many other fields of science. . . . The anxiety to publish quickly, if unreflectively, is reinforced by the reward system in research, which links grant's and promotion to people's publication records." (Maddox, 1993) In short, the Results section of papers is swelling while the Discussion shortens to a rehash of known middle of the road facts. The paradigm appears to be killing critical thought.

In reference to the work presented here, it is contemporary to explain how the data and results attained will aid in greater understanding of the field as a whole. While not
untrue, I am concerned with piling more data onto the ever more unwieldy paradigm without examine the paradigm itself. In today's explosion of neoplastic nomenclature, terms and definitions seemed to have taken on a life of their own. While it is not my intent to debauch the oncogene or tumor suppressor theories, I do believe that the content and intent of these ideas may be long overdue for reconsideration.

Cancer is undoubtably a disease of genetic alterations. Recent studies indicate the number of these alterations is in the range of five to ten—a number which is definitely different from the two cooperating oncogenes hypothesis or RB deletion hypothesis. Perhaps these inconsistencies can be remedied by examining the model systems utilized. In vivo tumor studies quote larger numbers of genetic alterations required than do in vitro studies, perhaps, because these two systems are not interconvertable. Technology seems in some ways to have outraced our abilities to observe. The majority of oncogene activity studies have relied upon gene transduction experiments, which are experiments laced with assumptions. One assumes that all recipient cells are equivalent, that gene insertion locations are irrelevant, and that the transduced cell's genome is static. Recent results undermine each of these assumptions.

All recipient cells are unlikely to be equivalent. In fact, oncogene transfection studies may actually select for the more transformed phenotype. That is, that cells farther along on the tumorigenic pathway may be more susceptible to additional changes, and what's more, they may be more demonstrative of phenotypic changes. In most assays, transformation is the expected endpoint, and therefore, it's detection should come as no
surprise. However, our inability to distinguish the mechanism of transformation may sometimes lend too much credence to an individual event. That is, single oncogenes may be cooperating with other events, and we simply can't detect it.

The whole tumor suppressor hypothesis negates the assumption that gene insertion events are irrelevant. Furthermore, tumorigenic phenotypes are far from static. These dynamic structures change and adapt rapidly to the immediate environment. What then of the terms "oncogene" or "tumor suppressor gene?"

The term oncogene is certainly a useful classification for genes involved in the tumorigenic process, but it is dangerous as a blinding definition. Cancer biologists often elucidate the workings of normal cell. Today I believe that greater understanding of the wild type gene is necessary to further illuminate the mechanisms of carcinogenesis. Every oncogene, be they cancer causing genes or not, has an endogenous function, and in the disturbance of that natural function cancer arises. The ability of oncogenes to be activated by mutation is puzzling evolutionarily. It seems to be an overly perilous predicament that natural selection should have absolved. If, however, activated oncogenes have an essential role in the development or maintenance of the organism, then their conservation is less mysterious. Activated oncogenes could for instance be part of a mechanism for rapid proliferation of a population of cells predestined to differentiate. A mechanism in a sense directly opposite of apoptosis. However, a role for activated oncogenes in normal homeostasis would even further cloud an already hazy definition.

Similarly, the nomenclature of "tumor suppressor genes" seems to be more useful
experimentally than functionally. To suppose that cells maintain tumor suppressor genes, supposes that they are prepared for cancer \textit{a priori}. More likely, these genes are members of a highly complex system that regulates normal development, proliferation, and differentiation. I propose that their role as tumor suppressing agents is an added advantage consistent with the notion that "normal development" excludes malignancy by definition.

In conclusion, it is not surprising that data accumulate faster than does understanding. However, I believe that moments like the attainment of a degree should symbolize not only the goal of a greater comprehension, but also the ongoing struggle to uncover the truth.

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