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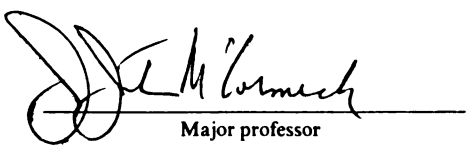

Major professor

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CLONING AND CHARACTERIZATION OF GENES ASSOCIATED WITH THE NEOPLASTIC TRANSFORMATION OF HUMAN FIBROBLASTIC CELLS

By

Jing Qing

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ABSTRACT

CLONING AND CHARACTERIZATION OF GENES ASSOCIATED WITH THE NEOPLASTIC TRANSFORMATION OF HUMAN FIBROBLASTIC CELLS

By

Jing Qing

To understand the genetic changes involved in the malignant transformation of human fibroblast cells, I carried out differential mRNA display analysis, comparing the infinite life span, karyotypically stable, non-tumorigenic human fibroblast cell strain MSU-1.1 and one tumor-derived cell line, designated 6A/SB1, which had been malignantly transformed by carcinogen treatment of MSU-1.1 cells. Five of the nine differentially expressed cDNA fragments identified by differential display were confirmed to be markedly downregulated in 6A/SB1 cells by Northern blot analysis. DNA sequencing followed by a computer search of databases indicated that two of these were unique. One of the cDNAs corresponds to *fibulin-1D,* and the other one corresponds to a novel gene, designated ST7. Northern and Western blotting analysis of 16 cell lines established from tumors formed in athymic mice by MSU-1.1-derived cell strains independently transformed in culture showed that 44% exhibited low level or lack of expression of fibulin-1D mRNA and protein. In a similar analysis of 15 malignant cell lines derived from patients, 80% showed low level or no expression of fibulin-1D. To study the role of fibulin-1D in transformation, I transfected 6A/SB1 cells and a human fibrosarcoma-derived cell line (SHAC) with a fibulin-1D cDNA expression construct. Transfectants displaying high levels of fibulin-1D were isolated and characterized. Elevated expression of fibulin-1D led to reduced ability to form colonies in soft agar and reduced invasive potential as tested in a matrigel in vitro invasion assay. Furthermore,

expression of fibulin-1D resulted in a markedly extended latency in tumor formation in athymic mice.

Using Northern analysis, I found that 10 out of 15 tumor cell lines derived from patients and 6 out of 16 cell lines established from tumors formed in athymic mice by MSU-1.1 cells transformed in culture have low or undetectable levels of ST7 mRNA. Molecular cloning of a near full-length cDNA revealed that the novel gene encodes a putative transmembrane protein composed of 859 amino acids: the N-terminal domain consisting of 492 amino acids including a 5-fold cysteine-rich repeat of 40 amino acids homologous to the ligand binding repeat of the known low density lipoprotein receptor, a 24 hydrophobic amino acid stretch spanning the plasma membrane, and a C-terminal domain of 343 residues. The ST7 gene is widely expressed in normal human tissues and is particularly abundant in human heart and skeletal muscle. Western blotting analysis using a specific anti-ST7 peptide antibody demonstrated that the levels of ST7 protein are high in normal fibroblasts and low in 12 sarcoma-derived cell lines tested. Altered expression of ST7 appears to be controlled at the transcriptional and posttranscriptional level.

DEDICATION

To my daughter, Julia Yaxin Wei

for the magical, purest smile you give me everyday

To my husband, Dong Wei

without whom the research wouldn't be so joyful

To my parents, Shuhua Qing and Hanguo Li

for their endless love and faith in me

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INTRODUCTION

It is generally recognized that cancer development in humans is a multistep process, in which a series of genetic and epigenetic events lead to the emergence of a clone of cells that have escaped normal growth control (Peto, 1977; Farber, 1984; Klein, 1987; Weinberg, 1989; Bishop, 1991; Hunter, 1991). Altered expression of proto-oncogenes and tumor suppressor genes as a result of mutations or other changes plays a pivotal role in the development and progression of tumors. For example, at least four or five genetic changes, including loss of function of tumor suppressor genes and activation of proto-oncogenes, are necessary for the development of colon carcinomas (Fearon and Vogelstein, 1990; Cunningham and Dunlop, 1996). In human breast cancers, mutations in a number of oncogenes, tumor suppressor genes and many other genetic loci are required before a cell becomes malignant (Jones et al., 1995).

Despite the vast increase in our knowledge of oncogenes and tumor suppressor genes associated with human neoplasia over the past 15 years, the molecular events leading to the formation of most types of human tumors are still not well understood. A variety of genetic lesions are found in tumors, including point mutation, deletion, DNA amplification and chromosome rearrangement (Salomon et al., 1991; Lasko et al., 1991). Genes involved in cancer affect the normal functions of many cellular processes, including cell proliferation, senescence, apoptosis, cell-cell and cell-matrix interactions, DNA repair, invasion and motility, angiogenesis, and others (Sager, 1997). Yet very few cancer-related genes affecting these processes have been identified in human cancers.

Therefore, to understand the multistep nature of tumor formation, it is necessary to identify the genes that are involved in the transition of non-tumorigenic cells to malignant cells and establish any correlation between the disruption their functions and the tumor phenotype.

The recent introduction of several in vitro transformation systems utilizing human cells in culture (Stoner et al., 1991; Reznikoff et al., 1993; McCormick and Maher, 1994; Rhim et al., 1994; Park et al., 1995) has facilitated the investigation of the cellular and molecular mechanisms involved in the multistep carcinogenic process. One of the advantages of these model systems is that they provide a means of dissecting the carcinogenic process under well-defined conditions. In our laboratory, transfection of the v-myc oncogene into a human neonatal foreskin-derived fibroblast cell line LG1 led to the establishment of a near-diploid, karyotypically stable, infinite life span human fibroblast cell strain MSU-1.1 (Morgan et al., 1991). MSU-1.1 cells are phenotypically normal and do not form tumors in athymic mice. Transfection of MSU-1.1 cells with an activated *ras* oncogene expressed at high levels (Hurlin et al., 1989; Wilson et al., 1990) or treatment of MSU-1.1 cells with chemical carcinogens such as (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) (Yang et al., 1992) or gamma irradiation (Reinhold et al., 1996), followed by selection of focus-forming cells, results in cells capable of forming tumors in athymic mice. However, the cellular genes responsible for the neoplastic transformation induced by these carcinogens remain poorly understood.

One way to identify critical genes associated with malignant transformation is to analyze the gene expression profiles in normal and cancer cells with the same genetic background. Conventionally this is achieved using subtractive hybridization (Wieland et al., 1990; Lee et al., 1991) or differential hybridization (Steeg et al., 1988). The

recently developed differential mRNA display method (Liang and Pardee, 1992; Liang et al., 1993) involves the reverse transcription of the mRNAs followed by PCR amplification. The amplified cDNA fragments corresponding to the 3' termini of mRNAs are separated on a DNA sequencing gel. The advantage of this method is that it allows one to display rapidly and simultaneously the expression of mRNAs from various phenotypically distinct variants. Therefore, genes that are overexpressed or downregulated can be identified at the same time.

The objectives of the present study were (1) to identify genes that are differentially expressed between MSU-1.1 cells and one fibrosarcoma-derived cell line, designated 6A/SB1, which had been malignantly transformed by carcinogen treatment of MSU-1.1 cells; (2) to determine whether the altered expression of identified genes is relevant in human tumor development in vivo by surveying the expression of the identified genes in tumor-derived cell lines from patients; (3) to clone the full-length cDNAs that correspond to the differentially expressed genes; (4) to define the biological function of the cloned gene(s) in tumor development by introducing the gene(s) into a proper recipient cell line and testing transformation-related phenotype. By identification, cloning and characterization of differentially expressed genes involved in the malignant transformation of MSU-1.1 cells, we should gain insight into the nature of the genetic changes underlying the neoplastic transformation of human fibroblasts in culture. We hope this information will provide useful diagnosis markers. It may also provide new approaches to therapy.

Chapter I of the thesis reviews the literature that supports the mutation theory of the origin of human cancer. The various methods utilized to identify and isolate cellular oncogenes and tumor suppressor genes and the biochemical functions of these genes are discussed. Also discussed in Chapter I is the evidence that supports the multistep

hypothesis of carcinogenesis. Chapter II consists of a manuscript that will be published in the journal *Oncogene*. It describes the research I carried out which shows that fibulin-1D, an extracellular matrix protein, is downregulated in 6A/SB1 cells and a large fraction of fibrosarcoma-derived cell lines. Elevated expression of fibulin-1D in 6A/SB1 cells and a human fibrosarcoma-derived cell line, SHAC, led to reduced ability to form colonies in soft agar and reduced invasive potential as tested in a matrigel in vitro invasion assay. Furthermore, expression of fibulin-1D resulted in a markedly extended latency in tumor formation in athymic mice. Chapter III consists of a manuscript that has been submitted to the journal *Oncogene*. It describes my research to identify and clone a novel gene, designated ST7, which is downregulated in 6A/SB1 and many other fibrosarcoma-derived cell lines. The deduced amino acid sequence suggests that ST7 encodes a putative transmembrane protein. Using a specific anti-ST7 peptide antibody, I demonstrated that the protein level of ST7 is high in normal human fibroblasts and low in sarcoma-derived cell lines.

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CHAPTER 1

LITERATURE REVIEW

A. Evidence that supports cancer is a genetic disease

It is generally recognized that cancer is fundamentally a genetic disease, arising from changes in the DNA. Several lines of evidence support this theme: the detection of damaged chromosomes in cancer cells, the recognition of hereditary predisposition to cancer, the connection between cancer susceptibility and impaired ability of cells to repair damaged DNA, the evidence that relates the mutagenic potential of substances to their carcinogenicity, and the persuasive power of identified alterations in cancer-related genes. Here I describe some of the evidence in favor of the mutation theory of the origin of human cancer.

1. Chromosomal abnormalities in cancer cells

In 1914, Boveri first formulated the somatic mutation hypothesis on the origin of human cancer (translation published in 1929). He proposed that the origin of cancer cells was due to the "wrongly combined chromosome complex occurring in a somatic cell and that this caused abnormal cell proliferation. The unlimited tendency to rapid proliferation in malignant tumor cells [could result] from a permanent predominance of the chromosomes that promote division...Another possibility is the presence of definite chromosomes which inhibit division...Cells of tumors with unlimited growth would arise if those inhibiting chromosomes were eliminated." (Boveri, 1929). He proposed further that this defect was passed on to all cellular descendants of the original cancer cell.

The first chromosomal abnormality definitively associated with human cancer is the Philadelphia chromosome found in the leukemia cells of more than 90% of patients with chronic myelocytic leukemia. The Philadelphia chromosome results from a reciprocal chromosomal translocation between chromosome 9 and 22. So far, more than 100 commonly occurring translocations have been observed in leukemias, lymphomas and solid tumors (Solomon et al., 1991; Rabbitts, 1994). The consistent association of specific translocations with particular disease types, particularly when present as the sole chromosome abnormality, has led to the realization that these rearrangements identify critical genetic loci in the oncogenic process.

In addition to chromosomal translocation, other chromosomal abnormalities found in cancer cells include chromosomal inversions, insertions, deletions, amplification and aneuploidy (Ruddon, 1995). Some of these aberrations occur consistently in certain specific cancer types. For example, deletions of 11p13 are found in Wilms' tumors (Riccardi et al., 1990), and chromosome 9 monosomy is found in bladder adenocarcinoma (Solomon et al., 1991). These lead to the hypothesis that a specific locus is involved in a key way in generating these malignancies. It has also been found that certain chromosomal aberrations are common to tumors of different cellular origin. For example, the 3p13-23 region is commonly affected by deletions in small cell carcinomas and adenocarcinomas of the lung, renal cell carcinomas and ovarian adenocarcinomas (Dal-Cin and Sandberg, 1989). Deletions of the 1p11-22 region are found in melanoma, breast adenocarcinomas, and malignant fibrous histiocytomas (Solomon et al., 1991). These data suggest that a common locus must be inactivated for these tumors to develop.

2. Familial cancers

Inherited cancers represent a small fraction, perhaps 1% to 3% of total human

cancers (Knudson, 1977). About 50 forms of hereditary cancers have been reported (Li, 1988; Birch, 1994). For example, dominant genetic inheritance accounts for about 40% of retinoblastoma and 20% to 40% of Wilms' tumor. Familial adenomatous polyposis (FAP) of the colon is another example of a cancer transmitted as a Mendelian-dominant trait, with about 80% penetrance. Colorectal cancer will eventually develop in nearly 100% of untreated patients with FAP (Fearon and Johns, 1992). The Li-Fraumeni syndrome represents another hereditary neoplasia. Members of these affected families may develop a variety of cancers, including sarcomas, breast cancers, brain cancers, and leukemia at an early age. The inheritance pattern is that of an autosomal dominant trait with high penetrance (Garber et al., 1991). Collectively, these diseases suggest that a number of inherited predisposing mutations contribute to the causation of these cancer. The defective genes responsible for some of these diseases have been identified and cloned. For example, the *Rb* gene is responsible for retinoblastomas (Friend et al., 1986; Lee et al., 1987), the *p53* gene for Li-Fraumeni syndrome (Srivastava et al., 1990), and the *APC* gene for FAP (Fearon and Johns, 1992).

3. Defect in DNA repair and predisposition to cancer

One of the most convincing piece of evidence for a causal relationship between genetic mutations and cancer in humans comes from the fact that the incidence of cancer in patients with DNA repair deficiencies is greatly increased (Lindahl, 1994). In patients with certain recessively inherited disorders, such as xeroderma pigmentosum (XP), ataxis telangiectasia, Fanconi's anemia, Bloom's syndrome, and hereditary nonpolyposis colorectal cancer (HNPCC), the frequency of specific tumors is significantly higher than in the general populations. Each of these disorders is characterized by the inability to repair specific kinds of physical or chemical damage to DNA.

XP is the most widely studied of the repair-deficient human diseases. Seven complementation groups of XP and one group that does not complement (XP variant) have been identified so far (Cleaver, 1990). These patients are characterized by extreme sensitivity of the skin to sunlight and usually suffer an age-specific incidence of skin cancer that is several thousand times higher than normal individuals. They typically develop multiple skin tumors that lead to death from metastatic squamous or basal cell carcinoma or malignant melanoma (Cleaver and Kraemer, 1989). All XP patients except XP variants are defective in nucleotide excision repair. Due to this defect, the cells of XP patients are less efficient in removing DNA lesions induced by DNA-damaging agents, for example, lesions caused by the ultraviolet light from the sun, and therefore are more prone to acquire genetic mutations than normal cells.

Another well studied disease is HNPCC. Recently it has been found that the majority of HNPCC cases can be attributed to a defect in any one of the multiple loci responsible for DNA mismatch repair, for example, the hMSH2 (Fishel et al., 1993), hMLH1 (Papadopoulos et al., 1994; Bronner et al., 1994), hPMS1 and hPMS2 (Nicolaides et al., 1994) genes. It has been proposed that the initial event in the development of tumors in HNPCC patients is the functional loss of a critical mismatch repair activity (Modrich, 1994), resulting in expansion or contraction of short nucleotide repeat sequences, which ultimately lead to mutations in cancer-related genes. For instance, the type II TGFβ receptor gene, which is involved in negatively regulating epithelial cell growth, is mutated in many HNPCC tumors with the mismatch repair deficiency (Markowitz et al., 1995). The mutations found were either insertion or deletions in simple repeated sequences, which is consistent with defective mismatch repair.

4. Carcinogens and mutagens

The evidence that chemicals can induce cancer in humans has been accumulating for

more than two centuries (Miller, 1978). Since most chemical carcinogens or their activated metabolites can react with DNA and cause mutations (Maher et al., 1968; Miller and Miller, 1981; Conney, 1982), the most plausible theory of carcinogenesis is that cancer is caused by genetic mutations. The mutagenicity of an agent can be assayed by various methods. For example, the Ames test (Ames et al., 1973; Maron and Ames, 1983) uses several strains of bacteria, *Salmonella typhimurium*, which are histidine auxotrophs and have poor nucleotide excision repair mechanism and an increased permeability to exogenously added chemicals. Using this system, Ames and his colleagues have estimated that about 90% of all carcinogens tested are also mutagens. Moreover, few non-carcinogenic agents show significant mutagenicity in this test system (McCann et al., 1975). Consistent with this mutation theory of carcinogenesis, chemical agents that cause genetic changes are frequently carcinogenic (Lawley, 1989). The extent of formation of some specific DNA adducts, for example, alkyl-O^6-guanine, has been shown to quantitatively correlate with mutagenicity and carcinogenicity of nitrosamines and similar alkylating agents (Frei et al., 1978; Swenson et al., 1986). Polycyclic hydrocarbons, such as benzo(a)pyrene and 3-methylcholanthrene cause mutations in cultured Chinese hamster V79 cells, as measured by induction of resistance to 8-azaguanine and ouabain, and the degree of mutagenicity was related to the degree of carcinogenicity of the chemicals in vivo (Huberman and Sach, 1974; 1976).

B. The genetic elements governing cancers: Oncogenes

1. Oncogene hypothesis

Much of our understanding of the molecular mechanisms involved in cellular transformation comes from studies of tumor viruses. In 1910, Payton Rous first isolated a transforming agent from a spontaneous sarcoma formed in a Plymouth Rock chicken.

He demonstrated that tumor extracts still had the transforming ability after being passed through filters designed to exclude bacteria (Rous, 1911). Very little progress occurred on the identification of the agent until the late 1950s, when electron microscopic techniques enabled investigators to identify the infectious agent as a virus (Bernhard, 1960). In the 1960s and 1970s, the development of techniques of molecular virology led to the isolation and cloning of many animal tumor viruses, including those from mouse, chicken, cat and monkey (Tooze, 1980 and Weiss et al., 1982). Molecular characterization of these viruses revealed that they could be classified into two groups: one group having a genome composed of RNA and the other group having a genome composed of DNA (Tooze, 1980; Weiss et al., 1982). The RNA tumor viruses, named retroviruses, have a small RNA genome. Genetic analysis showed that, for many of these retroviruses, a viral gene(s) conferred transformed phenotype, i.e., increased refractivity or rounding of cells, or both, to host cells in culture. For example, several groups isolated temperature sensitive mutants of Rous sarcoma virus (RSV) that could induce morphological transformation of chick embryo fibroblasts at 36^0C but not at the non-permissive temperature (Martin,1970; Friis et al., 1971; Kawai and Hanafusa, 1971). Golde (1970) and Vogt and colleagues (1970) showed that irradiation of certain strains of avian sarcoma virus resulted in the formation of viruses that retained the ability to grow but were no longer able to transform. Transforming genes were also found in DNA tumor viruses such as simian virus 40 and polyoma virus (Abrahams et al., 1975; Treisman et al., 1981; Bishop, 1985). These genes capable of inducing transformation were termed oncogenes (Bishop and Varmus, 1983). It was hypothesized that fragments of endogenous retrovirus genomes lay scattered throughout the mammalian genome as residues of ancient germline infections by retroviruses. These fragmented viral genes might be able to transform the cell, once

activated by specific stimuli like those known to turn on latent proviruses (Huberman and Todaro, 1969). Even though this hypothesis is wrong, it led to our current insights into the molecular mechanisms of cancer development.

2. Identification and isolation of oncogenes

2.1. Identification by homology with retroviral oncogenes

By differential hybridization between a transformation competent RSV strain and a transformation defective RSV strain, Bishop and Varmus and their colleagues purified complementary DNAs corresponding to the transforming gene of RSV (c-*src*) (Stehelin et al., 1976a). Using a *src* cDNA as a probe, they carried out hybridization experiments with the genomic DNA of transformed chicken cells as well as normal cells and found that sequences homologous to this cDNA were present in both types of cells (Stehelin et al., 1976b). Later it was found that in normal salmon, mouse, calf and human DNA, there exist DNA sequences homologous to *src* (Spector et al., 1978a). RNA sequences corresponding to *src* were found in the cellular RNA of normal and neoplastic chicken cells, indicating that the gene is not only present but is also transcribed in both normal and transformed cells (Spector et al., 1978b). Further work extended this finding to many other retroviral oncogenes (Varmus, 1989).

Searching for the cellular homologue of viral transforming genes was a fruitful way to uncover cellular oncogenes. Using the retroviral oncogenes as probes, more than twenty cellular oncogenes have been identified and cloned. A comparison of the cDNA sequence of the retroviral genes and intron-exon structure of the vertebrate genes makes it clear that these oncogenes were normal components of the genome, and that the retrovirus had transduced them (Bishop and Varmus, 1982). Analysis of multiple viral oncogenes and their homologues in animal genomes by DNA sequencing revealed that the viral genes often were mutated or altered substantially (Varmus, 1984; Bishop,

1985; 1987). The genes responsible for transformation in both naturally occurring and virus-induced tumors are designated oncogene, and their normal, unaltered cellular forms are called proto-oncogenes (Varmus, 1989; Bishop, 1991).

2.2. Identification by gene transfer

In 1972, Hill and Hillavo showed that the DNA of cells transformed by RSV-infection was able to induce transformation of chicken embryo fibroblasts in culture as a result of transfer of the RSV genome. This finding led to the hypothesis that the DNA from cells transformed by chemical carcinogens or from cancer cell lines might also be able to transform normal cells. Weinberg and colleagues were the first to demonstrate that DNA from chemically transformed mouse fibroblasts were able to induce morphological transformation (focus formation) of NIH3T3 cells, an established non-neoplastic mouse fibroblast cell line (Shih et al., 1979). In 1981, several research groups reported that DNA from certain human tumor cell lines can also transform NIH3T3 cells (Shih et al., 1981; Krontiris and Cooper, 1981; Perucho et al., 1981). Cloning strategies were designed to rescue the dominant-acting human transforming genes from the transformed NIH3T3 cells. One method took advantage of the different repetitive sequences found in human and mouse cells. Alu sequences are highly repeated sequences (10×10^5 copies per genome) interspersed in the human genome and are present on average about once every 5 kb. During DNA transfection, approximately 10-30 kb of genomic DNA from the human tumor cells is transferred and integrated into the genome of the mouse cells. The detection of Alu sequences in the transformed NIH3T3 cells indicates that a human sequence has been taken up by the cells and may be contributing to the transformed phenotype. To eliminate human DNA that has nothing to do with the transformed phenotype, genomic DNA from NIH3T3 transformants was again transfected into normal NIH3T3 cells. Multiple rounds of

transfection and isolation of transformed cells lead to the enrichment of human sequences required for transformation of NIH3T3 cells. After enrichment, a genomic library was constructed using the DNA from NIH3T3 transformants. The DNA insert of human origin was identified by hybridization with Alu sequences and tested for transforming ability. This strategy led to the cloning of the *ras* oncogene from the EJ human bladder carcinoma cell line (Shih and Weinberg, 1982). At the same time, the *ras* oncogene was similarly cloned from several EJ-related bladder carcinoma cell lines (Santos et al., 1982; Der et al., 1982; Goldfarb et al., 1982).

Many modifications of the transfection focus assay have been developed. One approach was to co-transfect tumor DNA with an antibiotic resistance marker gene. The drug-resistant clones were then selected in vitro, pooled and injected into athymic mice where tumor formation was monitored. This technique has revealed at least one new oncogene, mas (Young et al., 1986). Another approach makes use of an expression cDNA library instead of the fragmented genomic DNA. The cDNA library is constructed with mRNA from tumor cells and introduced into NIH3T3 cells to select for focus. Several potential oncogenes have been identified with this method (Miki et al., 1991; Chan et al., 1993; 1994).

Such cloning experiments carried out with a large number of human tumor cell lines have revealed a diverse assortment of oncogenes (Varmus, 1984; Bishop, 1987; Miki and Aaronson, 1995). This method, however, suffers from several limitations. Among the most serious is its reliance on a single mesenchymal-derived recipient cell line which may not be susceptible to transformation by oncogenes from other cell types or to oncogenes which confer a property already acquired by NIH-3T3 cells (Varmus, 1984; Hunter, 1991).

2.3. Identification by chromosome translocations

Specific chromosome translocations are often associated with distinct types of neoplasms (Heim and Mitelman, 1989; Soloman et al., 1991; Rabbitts, 1994). This observation implies that genes affected by the translocations might be instrumental in the genesis of the associated tumors. Based on chromosome translocations, a number of oncogenes have been identified. A good example is the cloning of the *bcl-2* oncogene from B cell lymphoma cells.

A common chromosomal abnormality that occurs in at least 90% of human follicular lymphomas is the reciprocal t (14;18) (q^{32} ; q^{21}) translocation (Yunis et al., 1982). Since it was known that the immunoglobulin heavy chain gene (IgH) resides in chromosome band $14q^{32}$, several groups independently carried out Southern blot analysis using the *IgH* gene as a probe to test whether any chromosome rearrangements occurred in the IgH locus. Once they confirmed that this locus was mediating the translocation, genomic libraries were constructed from the DNA of follicular lymphoma cells carrying the t (14;18) chromosomal translocation, and screened with an IgH specific probe to isolate DNA fragments containing the breakpoint (Tsujimoto et al., 1984; Cleary and Sklar, 1985). Subsequent chromosome walking experiments with chromosome 18 DNA fragments flanking the breakpoint ultimately led to the isolation of the novel oncogene, *bcl-2* (Tsujimoto et al., 1985; Cleary et al., 1986). This translocation resulted in the juxtaposition of the *bcl-2* gene with the *IgH* gene, placing the *bcl-2* upstream of the enhancer of the immunoglobulin gene and resulting in overexpression of the bcl-2 gene product (Graninger et al., 1987).

Indeed several of the known cellular oncogenes have been found adjacent to interchromosomal breaks. For example, the human c-*myc* cellular oncogene, normally located at chromosome $8q^{24}$, is juxtaposed with the heavy, κ, or λ immunoglobulin (Ig) genes from chromosome segments $14q^{32}$, $2p^{11}$, or $22q^{11}$ in the well characterized

Burkitt's lymphoma translocations (Taub et al., 1982; Dalla-Favera et al., 1983; Leder et al., 1983). These interchromosomal recombinations place the c-*myc* gene next to an Ig locus and result in enhanced expression of the c-*myc* gene (Erikson et al., 1983; Taub et al., 1984). Another example is the 9:22 Philadelphia translocation present in almost all cases of chronic myelogenous leukemia (deKlein and Hagemeijer, 1984). The translocated gene on chromosome 9 is the c-*abl* oncogene (deKlein et al., 1982). The translocation results in the deletion of the amino-terminal portion of the abl gene product and the replacement of it with the amino-terminal of the bcr (for breakpoint cluster region) gene (Heisterkamp et al., 1985). This fusion protein exhibits elevated tyrosine kinase activity (Konopka et al., 1984).

2.4. Identification by other routes

2.4.1. Identification by gene amplification

Many human tumors have amplified DNA sequences. The presence of amplified DNA sequences is often, although not always, manifested by the presence of chromosomes containing homogeneously staining regions (HSRs), or paired acentric chromatin bodies termed double minutes (DMs). Analysis of tumor cells and cell lines that exhibit DMs or HSRs has revealed the amplification of oncogenes. One such example is the isolation of the *mdm-2* oncogene.

A spontaneously transformed derivative of a mouse 3T3 cell line (designated 3T3-DM) contains an average of 25-30 DMs per cell. To isolate cDNA clones that represent genes amplified and overexpressed in the 3T3-DM cells, Cahilly-Snyder et al. (1987) constructed a cDNA library using mRNA from these cells. By differential hybridization with RNA from mouse cells lacking DMs or HSRs, they isolated two cDNAs representing amplified genes that were associated with the DMs, designated mdm-1 and mdm-2 (for mouse double minute). Later they demonstrated that enhanced

expression of the *mdm-2* gene could induce tumorigenicity in NIH3T3 and Rat 2 cells (Fakharzadeh et al., 1991).

2.4.2. Identification by structural homology

Several of the oncogenes identified as described earlier belong to multigene families. It is attractive to assume that genes homologous to known oncogenes may also be candidates for oncogenes. For example, using probes derived from the *c-myc* oncogene, three other related genes have been identified, i.e., N-*myc*, L-*myc* and R-*myc* (Depinho et al., 1987). Furthermore, it has been shown that all four *myc* genes have transforming activity (Schwab et al., 1985; Depinho et al., 1987). Another example is the cloning of ras-related genes. Ha-, Ki-, and N-*ras* genes belong to a closely conserved family. Using ras probes, at least five more distantly related genes have been identified. One of these, R-*ras* (Lowe et al., 1987), has been reported to be mutated in human ovarian carcinoma cell lines and the mutated gene, when introduced into NIH3T3 cells, induced a fully malignant phenotype (Saez et al., 1994).

3. Function of oncogene products

To date, more than 100 oncogenes have been identified. Extensive studies over the past two decades have revealed that proto-oncogene products are involved in the regulation of normal cellular growth and differentiation. Based on their biochemical functions, the protein products encoded by cellular oncogenes can be classified into seven groups. I will review examples in each category.

3.1. Growth factors as oncogenes

In 1983, two groups of investigators reported that the sequence of the v-*sis* transforming gene, the acutely transforming oncogene of simian sarcoma virus, was homologous to the gene encoding the B chain of the platelet-derived growth factor (PDGFB) (Doolitle et al., 1983; Waterfield et al., 1983). Since then, several oncogene

products have been found to be growth factors. For example, oncogenes *hst* (Delli et al., 1987) and *int-2* (Moore et al., 1986) encode fibroblast growth factor-related proteins.

In general, growth factor stimuli are transmitted into the cell via specific transmembrane receptors that modify key regulatory proteins in the cytoplasm. These in turn affect the decisions controlling cell proliferation and differentiation, including changes in gene expression and reactivity to other factors. The relationship between growth factors and malignant transformation was first suggested by Sporn and Todaro (1980) who proposed the autocrine model. This model proposes that growth factors and their cognate receptors are co-expressed by malignant cell populations, and that ligand-receptor interaction results in the autonomous proliferation of tumor cells. Several lines of evidence support this hypothesis. Expression of PDGF and its receptors has been reported in a high fraction of sarcomas as well as glioblastomas (Heldin and Westermark, 1989; Maxwell et al., 1990). Similarly, at least 70% of small cell lung cancer tumors and tumor-derived cell lines co-express the genes for stem cell factor and its receptor, the c-*kit* proto-oncogene (Krystal et al., 1996). Human colorectal carcinomas have been demonstrated to express a variety of growth factors, such as epidermal growth factor (EGF), transforming growth factor α (TGFα) and their cognate receptors, forming multi-autocrine loops (Shirai et al., 1995). These data suggest that aberrant production of secreted growth factors can play decisive roles in tumorigenesis by increasing the proliferation rate and degree of cellular autonomy (Aaronson, 1991; Cross and Dexter, 1991).

3.2. Protein tyrosine kinases as oncogenes

The association of tyrosine phosphorylation with malignant transformation was first suggested by the discovery that many of the viral oncoproteins catalyzed the phosphorylation of proteins on tyrosine residues, and the level of phosphotyrosine in

proteins is elevated as much as 10-20 fold in cells transformed by these oncogenes (Hunter, 1987). To date, more than 40 protein tyrosine kinases have been identified. They can be classified into two groups: (1) Receptor protein tyrosine kinases, which span the plasma membrane with large extracellular and cytoplasmic domains; (2) cytoplasmic non-receptor protein tyrosine kinases, many of which are attached to the plasma membranes. I will describe an example for each category.

3.2.1. Receptor protein tyrosine kinases: the EGF receptor as the prototype

Because growth factor receptor tyrosine kinases have the ability to generate a mitogenic signal, these molecules possess a latent oncogenic potential which, when activated, results in unregulated cell growth. In fact, several oncogenes are homologues of receptor tyrosine kinase. For example, the v-*erbB* oncogene is derived from the chicken EGF receptor gene (Yarden and Ullrich, 1988). The v-*fms* oncogene is derived from the gene for the receptor for the colony-stimulating factor 1 (Sherr, 1990).

The EGF receptor (EGFR) is a 170 kDa transmembrane tyrosine kinase that is expressed on a wide variety of cell types. Other members in the EGFR family include p185neu tyrosine kinase (also known as HER2 or c-erbB-2), erbB-3 and erbB-4 (Schlessinger and Ullrich, 1992; Plowman et al., 1993). All four receptors possess a glycosylated, cysteine-rich, extracellular ligand binding domain, a single hydrophobic transmembrane region, and a cytoplasmic domain that contains a tyrosine kinase catalytic domain (Ullrich and Schlessinger, 1990).

The interaction of a ligand with the EGFR or the p185neu/HER2 results in receptor dimerization, autophosphorylation (Ullrich and Schlessinger, 1990), and enhanced tyrosine kinase activity toward other substrates which elicit a mitogenic response in EGF-sensitive cells. Certain signaling molecules become physically associated and/or phosphorylated by the activated EGFR kinase. Those identified include the

phosphatidylinositol (PI)-specific phospholipase C-γ (PLC-γ) (Rotin et al., 1992; Vega et al., 1992), the PI-3'- kinase (PI3K), the PI4 kinase and the PI5 kinase (Cochet et al., 1991), the GTPase activating protein (GAP) (Margolis et al., 1990), and the adaptor protein Shc (Pelicci et al., 1992). PLC-γ is involved in the generation of two important messengers, inositol triphosphate and diacyl glycerol (Berridge and Irvine, 1989; Kikkawa et al., 1989). The former causes release of stored intracellular calcium and the latter activates protein kinase C (PKC). These secondary messengers appear rapidly in cells following stimulation with growth factors such as EGF. PKC belongs to a multigene family that encodes at least ten distinct isoforms (Nishizuka, 1992). Overexpression or gene alteration of certain isoforms of PKC has been reported to increase cell proliferation in culture (Housey et al., 1988; Hsiao et al., 1989) and induce neoplastic transformation (Cacace et al., 1993). The actions of a number of tumor promoters are thought to be mediated by PKC (Berridge and Irvine, 1989; Kikkawa et al., 1989). The importance of the PI3 kinase in transformation has been underscored by the recent identification of a new avian sarcoma virus, ASV16. The oncogene carried by ASV 16 encodes a Gag-PI3K p110 catalytic subunit fusion protein, which has PI3K activity (Hunter, 1997; Chang et al.,1997). The adaptor protein Shc has been shown to be a potent transforming gene when overexpressed (Pelicci et al., 1992).

Constitutive activation of receptor protein tyrosine kinases can be achieved in a number of ways. In the case of the v-*erbB* oncogene, deletion of the extracellular ligand binding domain eliminates the negative control that this structure normally exerts on the cytoplasmic domain. A single point mutation in the p185neu transmembrane domain results in constitutive receptor oligomerization and activation (Weiner et al., 1989). Receptor-derived oncogenes possess other structural lesions such as point mutations and deletions in the cytoplasmic region, and carboxyl-terminal truncations that appear to

enhance and modulate the transforming signal (Woolford et al., 1988; Khazaie et al., 1989). However, these mutations seem to play a minor role in human cancer, since the most common cellular lesion found in human cancers involves autocrine activation (see B.3.1) in association with receptor overexpression. Members of the EGFR family have been shown to be amplified or overexpressed in human tumors of several types, including lung cancer, breast cancer, ovarian cancer (Veale et al., 1987; Slamon et al., 1989), head and neck cancers (Grandis and Tweardy, 1993), glioblastomas (Nishikawa et al., 1994) and bladder cancer (Lipponen and Eskelinen, 1994).

3.2.2. Nonreceptor protein tyrosine kinases: Src as the prototype

As noted above, *Src* was the first transforming oncogene discovered. At least nine members of the *Src* gene family have been discovered (Bolden et al., 1992; Brown and Cooper, 1996). Several were initially discovered as the normal counterparts of viral oncogenes. These proteins share structural similarities. They all have an extreme N-terminal myristoylation signal presumably required for their attachment to the plasma membranes, the Src homology (SH) 3 and SH2 regions, a kinase domain, and a C-terminal regulatory tail.

The normal cellular functions of Src are still not clear. It has been shown that Src kinase is activated in PDGF-, CSF1-, EGF-, and FGF-stimulated fibroblasts (Brown and Cooper, 1996), and associates with the PDGF and CSF-1 receptors through its SH2 domain (Alonso et al., 1995). Activated Src protein has been shown to phosphorylate proteins that are implicated in mitogenic signaling pathways such as PI-3 kinase (Gutkind et al., 1990; Haefner et al., 1995), GAP protein (Brott et al., 1991), and the adaptor protein, Shc (McGlade et al., 1992; Egan et al., 1993). These findings provide a biochemical link between the Src-family kinases and pathway utilized by activated growth factor receptors and suggest a role for Src in growth control. Therefore, v-Src

and activated c-Src may transform cells by upregulating mitogenic signaling pathways normally stimulated by growth factors.

Another class of Src kinase substrates that have been identified includes proteins involved in cytoskeletal organization (e.g., vinculin), cell-substrate adhesion (e.g., fibronectin receptor), and cell-cell adhesion (e.g., focal adhesion kinase) (Reviewed by Brown and Cooper, 1996). The functional significance of phosphorylation of these proteins has yet to be established.

3.3. Membrane associated G-proteins as oncogenes

Two forms of membrane associated G-proteins have been implicated in the regulation of cellular proliferation (Bourne et al., 1990): the heterotrimeric G proteins and the monomeric products of the *ras* and related genes, including Ha-, Ki-, and N-*ras*. Recently, two more ras-like genes, *TC21* and R-*ras* have become potential targets for mutational activation in human cancers (Saez et al., 1994; Hunter, 1997).

The normal function of ras family proteins is to serve as an ubiquitous signal transducer for multiple growth factor receptor tyrosine kinases (Crews and Erikson, 1993; Egan and Weinberg, 1993; McCormick, 1993; Marshall, 1995; Seger and Krebs, 1995). When a ligand such as EGF or PDGF binds to its receptor, the receptor is activated, triggering an autophosphorylation event. This leads, through intermediates such as adaptor proteins Shc and Grb2 (for growth factor receptor binding) to the guanine nucleotide exchanging factor Sos (for son of sevenless), resulting in recruitment of Sos to the plasma membrane where ras is bound. The interaction between the guanine nucleotide releasing activity of Sos and ras leads to the release of GDP and binding of GTP to ras (Feig, 1993). This activates ras, which in turn leads to activation of a kinase cascade including Raf, Mek (also known as mitogen-activated protein kinase kinase or MAPKK), and MAP kinase (MAPK). The substrates of MAPK include various

molecules associated with cell growth control, including transcription factors, enzymes, and regulators of protein synthesis (Marshall, 1995).

This signal transduction pathway is a crucial pathway regulating growth and differentiation in many cell types. In fact, mutations in the *ras* family of oncogenes are the most frequently detected alterations in oncogenes in both animal tumor model systems and in human cancers (Bos, 1989). Oncogenic forms of *ras* genes differ fromtheir normal counterparts by having a single mutation in codon 12,13, 59 or 61, which reduces their intrinsic GTPase activity and their ability to interact with GAP, thus keeping p21ras in the GTP-bound, activated mode (Tong et al., 1989; Krengel et al., 1990).

Besides this well characterized ras-Raf-MAPK pathway, it has recently been shown that ras activation of Raf-MAPK independent pathways is sufficient to cause tumorigenic transformation (Khosravi-Far et al., 1996). Among the targets for *ras* required for transformation is Rac, a member of the Rho family of small G proteins. Mutant Rac has modest transforming activity, and a dominant negative mutant Rac blocks ras induced transformation (Khosravi-Far et al., 1995; Qiu et al., 1995).

3.4. Ser/Thr kinases as oncogenes: Raf-1 as the prototype

Raf-1 was first identified as the cellular homologue of the viral oncogene v-*raf*. The *Raf* family of oncogenes encodes serine/threonine-specific protein kinases. It comprises three members: Raf-1, A-Raf and B-Raf (Storm et al., 1990; Rapp, 1991). The three genes are dispersed over different chromosomes and have been mapped to sites that are frequently altered in human tumors (Storm et al., 1990). All three genes can be converted into oncogenes by N-terminal fusion, deletion or site-specific mutations (Rapp, 1991; Storm and Rapp, 1993). Raf proteins are believed to be key components of the growth factor receptor-ras-MAPK mitogenic signal transduction pathway (See

B.3.3.). Raf proteins can also be activated by phorbol ester through protein kinase C (Rapp, 1991; Stephens et al., 1992) or by G-protein coupled receptors (Cook et al., 1993; Howe and Maeshall, 1993).

A number of studies also suggest that Raf-1 can elicit its oncogenic effect independent of MAPK activation. In Rat-1 cells, expression of a transfoming, activated Raf-1 does not stimulate MAPK activity and yet is sufficient to induce transformation (Gallego et al., 1992; Samuels et al., 1993). The mechanisms underlying the transformation are not clear.

Raf-1 may also positively regulate cell growth by promoting progression of the cell cycle. It has been shown that Raf-1 can phosphorylate and activate the cdc25 phosphatases (Galaktionov et al., 1995).

3.5. Nuclear transcription factors as oncogenes

Modulation of gene expression is frequently an important ramification of intracellular signaling and plays a vital role in the control of cell proliferation and differentiation. A number of nuclear transcription factors and transcription regulators have been implicated in different types of cancer (Lewin, 1991; Forrest and Curran, 1992). One example is the activator protein 1 (AP-1).

AP-1 serves as the nuclear target of many oncogenic signal transduction pathways (Angel and Karin, 1991; Treisman, 1994). This multigene group includes members from the Fos (c-fos, fosB, fra-1, and fra-2) and Jun (c-jun, jun B, and jun D) families. Members of the Fos family proteins form heterodimers with Jun proteins via their bZip (basic region plus leucine zipper) regions. The complex binds to specific DNA sequences and regulates the transcription of genes containing the cis-acting element in their promoters.

Both Fos and Jun were initially identified as retroviral oncogenes (Curran et al., 1982;

Maki et al., 1987). In keeping with their role in the control of cell proliferation, expression and activity of these genes are transiently stimulated by mitogenic signals. Rapidly dividing cells show increased levels of AP-1 transcriptional activation (Vogt, 1994). Studies using loss-of-function approaches, i.e., the expression of antisense RNA or the microinjection of antibodies showed that AP-1 complexes are required for theinitiation of DNA synthesis during S phase (Holt et al., 1986; Carter et al., 1991; Kovary and Bravo, 1991; Smith and Prochownik, 1992). This function may be mediated by regulating the transcription of cyclin D1 (Albanese et al., 1995; Phuchareon and Tokuhisa, 1995). Studies using transgenic mice showed that stable expression of c-fos led to dysregulation of bone growth, eventually resulting in osteosarcomas and chondrosarcomas (Ruther et al., 1987; 1989; Wang et al., 1991). Transgenic mice expressing an oncogenic form of jun developed fibrosarcomas at sites of wound healing (Schuh et al., 1990). A recent study with c-fos-null mice (Saez et al., 1995) demonstrated that c-fos is necessary for progression from benign papillomas to malignant squamous cell carcinomas and spindle cell tumors. Furthermore, activation of AP-1 activity has been documented in several types of human cancer, including human squamous cell carcinoma of the lung (Volm et al., 1992; Wodrich and Volm, 1993) and breast cancer (Walker and Cowl, 1991). Collectively, these studies demonstrate that altered activity of AP-1 complex could affect mitogenic control and promote neoplastic transformation of cells in specific tissues.

However, AP-1 is not connected exclusively to positive growth signals (Sassone-Corsi, 1992). In a number of systems increased AP-1 activity is correlated with cessation of cell growth and differentiation (Vogt, 1994). The role of AP-1 as a growth stimulator or a growth attenuator is determined by additional, largely unknown factors.

3.6. Cell cycle kinase activators as oncogenes

In eukaryotic cells, progression through the cell cycle is strictly controlled by a family of cyclin proteins, the cyclin-dependent kinases (cdks), and kinase inhibitor proteins (Hartwell and Kastan, 1994; Hunter and Pines, 1994; Nurse, 1994). Unique combinations of cyclins and cdks assemble during each phase of the cell cycle. Theassociation of cyclins and cdks allows the subsequent activation of the complex and drives cell proliferation forward by phosphorylating specific substrates.

The control of mammalian cell proliferation by extracellular signals occurs largely during the G1 phase of the cell cycle (Hunter and Pines, 1994; Hall and Peters, 1996). During the G1 phase, cells respond to extracellular signals by either advancing toward another division or withdrawing from the cycle into a resting state (G_0) (Pardee, 1989; Sherr, 1994). Unlike transit through the S, G2, and M phases, G1 progression normally relies on stimulation by mitogens and can be blocked by antiproliferative agents. The D type cyclins (D1, D2, and D3) and cyclin E are the primary G1 phase cyclins in mammalian cells (Sherr, 1993). Expression of the D type cyclins is rapidly induced after exposure of cells to mitogens; this expression declines when mitogens are withdrawn or antiproliferative agents are added (Sherr, 1994). Thus one might expect that the deregulation of cyclin D synthesis would make cell cycle progression less dependent on growth factors and contribute to oncogenesis.

Recent studies have shown that, as a group, mutations in or deregulated expression of cell cycle genes constitute the most common genetic changes in tumor cells (Sherr, 1996), especially in one or another of the genes involved in controlling progression through the G1 phase of the cell cycle. The best characterized one is cyclin D1.

Cyclin D1 is encoded by the *CCND1* gene on chromosome 11q13 (Xiong et al., 1992). It was originally cloned as an oncogene, termed *PRAD1*, which was found to be activated by inversion of chromosome 11 in the genome of parathyroid adenoma cells

(Motokura et al., 1991; Rosenberg et al., 1991a). This inversion places *CCND1/PRAD1* gene under the control of the parathyroid hormone gene promoter, resulting in overexpression of cyclin D1. In centrocytic lymphoma and multiplemyeloma, overexpression of cyclin D1 is achieved by a reciprocal translocation of chromosome 11q13 and the IgH locus on 14q34, bringing cyclin D1 gene under the influence of the IgH enhancer (Seto et al., 1992; Rosenberg et al., 1991b). Amplification and overexpression of cyclin D1 have also been documented in a broad spectrum of common human cancers, including breast cancers, squamous cell carcinomas of the head and neck, bladder cancers, small cell lung cancers and esophageal carcinomas (Hall and Peters, 1996). Consistent with the oncogenic role of cyclin D1 are observations that transgenic mice engineered to overexpress this cyclin in their breast tissues are prone to mammary adenocarcinomas (Wang at al., 1994), and that co-expression of cyclin D1 and myc genes in the lymphoid tissues of transgenic mice leads to rapid development of lymphomas (Bodrug et al., 1994; Lovec et al., 1994).

3.7. Inhibitors of apoptosis as oncogenes

In the past few years, accumulating evidence suggests that an imbalance of homeostasis between cell growth and death plays a role in cancer development. Cancer cells usually acquire the ability to escape from programmed cell death (apoptosis) in response to DNA damage or other suboptimal conditions that would induce a normal cell to die. Bcl-2 family proteins are key regulators of apoptosis and are the best candidates implicated in carcinogenesis.

As discussed earlier (see B.2.3), the *bcl-2* gene was first identified as an oncogene involved in the development of human follicular lymphoma (Tsujimoto et al., 1984). The t(14;18) translocation brings the *bcl-2* gene under the control of the IgH enhancer, resulting in constitutively high expression of bcl-2 protein (Graninger et al., 1987).

The frequent association of a translocation involving the bcl-2 locus with cancer suggests that the *bcl-2* gene may be an oncogene. Introduction of bcl-2 into aninterleukin 3-(IL-3)-dependent cell line led, not to factor-independent growth, but to factor-independent survival, and the cells were not tumorigenic (Vaux et al., 1988). Reed et al. (1988) reported that 3T3 cell constitutively expressing bcl-2 proteins only rarely became transformed. These studies were interpreted as indications that bcl-2 can contribute to tumorigenesis by allowing cells to survive, but malignancy requires additional genetic changes.

Consistent with this view, the generation of bcl-2 transgenic mice using the IgH enhancer demonstrated that in the B cell lineage, deregulated cell survival resulted in dramatic polyclonal expansion of mature B lymphocytes and the development of diffuse immunoblastic B cell lymphomas. A high proportion of these tumors had rearrangements of the c-*myc* gene as a second genetic alteration (Strasser et al., 1990; McDonnell and Korsmeyer, 1991; Katsumata et al., 1992). Targeted overexpression of the *bcl-2* gene in thymic lymphocytes, mammary and intestinal epithelium, and myeloid populations has not produced tumors (Hockenbery, 1994).

One model for the function of the *bcl-2* gene in carcinogenesis proposes that the *bcl-2* gene acts synergistically with specific oncogenes to inhibit cell death under adverse growth conditions. This concept follows the recognition that several oncogenes have dual effects on cell proliferation and cell death. For example, expression of the c-*myc* gene promotes cell proliferation when growth conditions are favorable. However, when cells enter a growth arrest phase due to absence of growth factors or the action of cytostatic drugs, expression of c-myc induces apoptosis (Packham and Cleveland, 1995). C-myc-induced apoptosis has been shown to be blocked by overexpression of bcl-2 (Bissonnette et al., 1992; Evan et al., 1992; Fanidi et al., 1992). Additional cellular

oncogenes that can predispose cells to undergo apoptosis have been found, including c-*fos* and c-*rel* (Smeyne et al., 1993; Abbadie et al., 1993).

Deregulation of the bcl-2 gene may also function as a survival mechanism in cancer cells predisposed to cell death by environmental factors. Bcl-2 acts as a broad anti-apoptotic factor and opposes cell death following treatment with ionizing radiation and cancer drugs as well as hormone manipulations (Sentman et al., 1991; Miyashita and Reed, 1993). Several non-lymphoid neoplasms, including lung, prostate, colon and breast cancers, express high levels of bcl-2 protein (McDonnell et al., 1992; Pezzella et al., 1993; Bronner et al., 1994).

C. The genetic elements governing cancers: Tumor suppressor genes

1. Tumor suppressor gene hypothesis

The existence of tumor suppressor genes that can suppress or inhibit the growth of tumor cells is supported by three lines of evidence: (1) studies on somatic cell hybrids between normal and tumor cells; (2) familial cancer syndromes; and (3) the loss of specific chromosomes in cancers. I will focus on studies on somatic cell hybrids and familial cancer syndromes.

1.1. The suppression of malignancy in cell hybrids

Somatic cell hybrid experiments provided the first evidence that genetic changes underlying the neoplastic transformation might result from the loss of function of specific genes. In these experiments, tumorigenic cells were fused with nontumorigenic cells, and the resulted hybrid cells were nontumorigenic (Harris et al., 1969; Harris, 1988). However, hybrids passaged in culture for extended periods were sometimes tumorigenic. Careful studies demonstrated that the tumorigenic segregants had lost one or more chromosomes from the initial hybrids (Harris and Klein, 1969; Stanbridge et al., 1981; Evans et al., 1982), and that suppression of malignancy in the hybrid cells

required the retention of specific chromosomes derived from the normal cells (Stanbridge et al., 1981; Klinger, 1982; Harris, 1988). This phenomenon of suppression holds true for a wide variety of cell or tissue types (Sager, 1985; 1989; Levine, 1993). Taken together, these experiments suggest that recessive genetic changes can be responsible for the tumorigenic phenotype and that gene products of normal alleles from normal cells are able to suppress tumorigenicity.

By analyzing chromosomal loss in the tumorigenic variants of suppressed hybrids and using a microcell transfer technique, in which only one single chromosome derived from the normal cells is transferred to tumorigenic cells, experiments have identified and mapped the specific chromosomal location of some of these suppressor genes (Marshall, 1991; Levine, 1993).

1.2. Familial cancer syndromes: Knudson's hypothesis

A small proportion of most types of human tumors occur in familial form (Knudson, 1977; Li, 1988). Several well-defined tumor syndromes are inherited as simple autosomal dominant traits with high penetrance (Birch, 1994). Retinoblastoma, a rare pediatric eye tumor, is the prototype of this group.

From the epidemiological studies of retinoblastoma, Knudson (1971) noted that 40% of retinoblastoma cases occurred in young children with a mean age of 14 months, and these tumors were often bilateral (originating in both eyes) with the mean number of tumors being three. In some cases there was a family history of this disease. If these patients were cured through surgical intervention, they often had a substantially increased risk of developing other malignancies, especially osteosarcoma. These observations suggested an inherited component for these cancers. Howevr, about 60% of the retinoblastoma cases didn't fit this pattern. In these cases, no familyhistory of cancers was noted. These tumors were typically first detected at a later age, and were

always unilateral (affecting only one eye) with one cancer per patient. This class of retinoblastomas was quite rare, occurring in about one in 30,000 people.

Following the earlier suggestions by De Mars (1970), Knudson (1971) proposed a hypothesis to explain the origins of these two categories of retinoblastoma. He suggested that children who develop retinoblastomas at an early age inherited a mutant allele of a gene, later called the retinoblastoma susceptibility gene or *Rb*, and that a second somatic mutation in this gene in a retinoblast would then give rise to this cancer. This was a common event, which was consistent with an average of three independent cancers per patient and occurred in 90% of such patients. In contrast, children who develop retinoblastoma at a later age did not inherit a mutant Rb allele, and two independent mutations of this gene in a single retinoblast must have occurred to give rise to a tumor since the *Rb* gene is recessive at the cellular level. This was consistent with the low frequency (one in 30,000 people). Predisposition to retinoblastoma was autosomally dominant because at typical mutation rates almost everyone who has inherited one defective *Rb* gene will sustain a second mutation in the *Rb* gene in at least one retinoblast. This unifying hypothesis leads to the concept of genes whose product normally prevents cancerous growth and both alleles must be lost via mutations for it to play a causal role in cancer development.

2. Identification and isolation of tumor suppressor genes

2.1. Identification by gene transfer

Unlike the dominant oncogenes, which can be identified with relative ease, assaying directly for tumor suppressor gene function is difficult. It is easy to identify a small number of transformed cells admist a background of normal cells because thetransformed cells have a growth advantage. In contrast, identifying a small number of normal (revertant) cells which grow like normal cells in a background of transformed

cells is not a trivial task. Based on observations that alteration in certain phenotypes in vitro is associated with loss of malignancy in vivo, several groups devised assays to clone tumor suppressor genes directly using a gene transfer technique (Noda, 1990).

In the course of characterizing the in vitro phenotypes of spontaneously nontumorigenic revertants from the v-Ki-ras-transformed NIH3T3 cells, Noda and colleagues found that flat revertants exhibited greatly reduced malignancy in vivo (Noda et al., 1983; Basin and Noda, 1987). They used "flatness" as an in vitro marker to clone tumor suppressor genes. They transfected the tumorigenic DT cell line (a subline of v-Ki-ras-transformed NIH3T3 cell) with a normal human foreskin fibroblast expression library, isolated flat revertants, and recovered the transfected cDNA, Krev-1, from the genome of the revertant cells. When Krev-1 was introduced into DT cells and expressed at high levels, some of the transformed phenotypes, e.g., the efficiency of colony formation and the size of the colonies in soft agar, were reduced. The Krev-1 gene encodes a protein homologous to the p21ras protein (Kitayama et al., 1989).

Since H-ras transformed rat FE-8 cells showed an increased sensitivity toward ouabain when compared to their normal counterparts (Noda et al., 1983), Schaefer et al. (1988) established a functional assay to identify and clone DNA sequence capable of suppressing neoplastic transformation. Genomic DNA from normal human placenta was introduced into FE-8 cells by co-transfection with a plasmid conferring drug resistance. Drug-resistant cells were subjected to treatment with ouabain. The surviving clones lost the morphology of transformed cells, acquired the ability to grow in an anchorage-dependent manner only, and showed reduced ability to form tumors in athymic mice.Using the human Alu repetitive sequences as probes, the suppressor gene in a secondary revertant clone was isolated.

These cloning experiments are largely based on alterations in certain phenotypes in

vitro that correspond well to loss of malignancy in vivo. The problem is lack of an efficient selection procedure. Also, the spontaneous revertants will give rise to false positives. Because of these limitations, gene transfer is not an efficient method to clone tumor suppressor genes.

2.2. Identification by subtractive hybridization, differential hybridization and differential mRNA display

Since malignant transformation is caused by accumulation of diverse genetic changes, including mutations and/or altered expression of critical genes (Sager, 1997), gene transfer experiments may not detect genes that are required but are not sufficient to suppress the malignant phenotype. Therefore, identification and cloning of genes differentially expressed between tumor cells and well matched normal counterparts, especially those genes that are downregulated in the tumor cells, may uncover new tumor suppressor genes. Currently, several methods are available for this purpose, namely, subtractive hybridization, differential colony hybridization and differential mRNA display.

Subtractive hybridization is designed to select for genes expressed uniquely or preferentially in one of a pair of closely related cell populations (Sargent, 1987). cDNA synthesized from the mRNA of one cell population is hybridized with excess amount of mRNA from the other cell population. The cDNA-RNA hybrids representing mRNAs that are equally expressed in both cell populations are efficiently separated from the unpaired cDNAs by hydroxyapatite chromatography. The unpaired cDNAs from the first round of hybridization are hybridized again with excess amount of mRNA from the other cell population. Usually the unpaired cDNAs from the second round of hybridization represent mRNAs that are uniquely expressed in the cell of interest. These cDNAs are inserted to a vector to construct a subtractive library or are used as probes to screen a

library. Using such a strategy, Sager and colleagues isolated several putative tumor suppressor genes involved in the development of breast cancer (Lee et al., 1991).

The technique of differential colony hybridization has been widely used to identify mRNAs regulated by cell differentiation, growth and cytokine treatment (Leonard et al., 1987; Sarma et al., 1992; Fernandez-Pol et al., 1993; Green et al., 1995). To identify genes uniquely expressed in metastatic cells, Steeg et al. (1988) utilized a series of related murine melanoma cell lines of varying metastatic potential. These cell lines are derived from K-1735 cell line. A cDNA library was constructed using mRNA from K-1735 cells. Duplicate filters of the library were prepared and one was hybridized to labeled cDNA from a low-metastatic cell line and one to cDNA from a high-metastatic cell line. Using this approach, they isolated a novel gene, termed *nm23*, whose expression level was inversely correlated to tumor metastatic potential (Sobel, 1990). The major drawbacks of this techniques are poor sensitivity and difficulty in isolating the relatively few positive clones from the high background hybridization (Cochran et al., 1987).

The differential mRNA display procedure developed by Liang and Pardee compares the profile of gene expression in closely related eukaryotic cells by systematically amplifying the 3' end of mRNAs using reverse transcription and PCR (Liang and Pardee, 1992; Liang et al., 1993). It consists of two steps. First, a subset of the mRNA populations are reversely transcribed using an anchored oligo(dT) primer; secondly, the3' end of mRNAs are amplified by PCR using an arbitrary 10mer primer and an anchored oligo(dT) primer. The PCR products are separated on a DNA sequencing gel. By comparing the band patterns from two cell populations, mRNAs that are upregulated or downregulated can be identified simultaneously. Compared with the conventional subtractive or differential hybridization method, differential mRNA display provides a

rapid and efficient approach to detect differentially expressed mRNAs from pairs of phenotypically distinct cells of the same genetic background. Using this approach, Sager and colleagues have isolated more than 50 novel genes which are no longer expressed or are strongly downregulated in breast carcinoma cell lines (Sager, 1997). Genes that are downregulated in other types of cancer have also been identified with this method (Sun et al., 1994; Simon et al., 1996).

2.3. Identification by positional cloning

A powerful way to identify and clone tumor suppressor genes is positional cloning, whereby a gene associated with certain types of cancer is isolated on the basis of its approximate chromosomal position (Collins, 1991; Wicking and Williamson, 1991). The first step in this approach is the mapping of the gene to a particular human chromosome. Then various strategies can be used to identify and characterize the specific gene in that genomic region. I will describe the various methods available for localization and cloning of such genes.

2.3.1. Localization of putative tumor suppressor genes

2.3.1.1. Localization by cytogenetics

Cytogenetic studies in solid tumors have generally yielded a plethora of inconclusive data but there have been some consistent findings that have led to the isolation of new tumor suppressor genes. For example, about 3% to 5% of hereditary retinoblastomacases were found to be associated with gross abnormalities in chromosome 13 (Gallie et al., 1990). Other studies found patients who had small interstitial deletions of 13q (Franke, 1978; Yunis and Ransay, 1978). In all cases studied, the deletion involved band 13q14.1. This regional deletion was also demonstrated in some sporadic retinoblastomas (Balaban et al., 1982). These studies suggested that the putative Rb tumor suppressor gene resided within 13q14.1.

Cytogenetic studies also helped to localize the tumor suppressor gene of Wilms' tumor on chromosome 11p13 (Riccard et al., 1990). However, most of the time, the regional chromosome loss is too small to be detected at the visible chromosome level. Molecular cytogenetics provides a more sensitive method to detect chromosome abnormality by analysis of extracted DNA.

2.3.1.2. Molecular cytogenetics: Loss of heterozygosity

Loss of heterozygosity (LOH) involves comparing normal and tumor tissues from the same patient to detect allele imbalance at polymorphic loci. The typical situation is that the two alleles from the non-tumorigenic cells of the body contain a restriction fragment length polymorphism (RFLP), while the cells from the tumor tissue have lost one of these two alleles. By using panels of RFLPs that map to different regions of a given chromosome, and studying LOH in enough tumors, a common region of deletion can be identified. LOH could arise from a loss of a chromosome, a small deletion of the genetic locus, or a gene conversion by homologous recombination. Typically a mutation arises in a tumor suppressor gene and this is followed by LOH at that locus, thus eliminating both wild-type alleles.

Besides RFLPs, microsatellite sequences are particularly informative for detection of LOH. They are extremely polymorphic so that most individuals are constitutionally heterozygous (Weber, 1990). Furthermore, they can be detected by PCR and are not dependent on the presence or absence of a restriction enzyme site.

2.3.1.3. Molecular cytogenetics: Linkage analysis

For those genes associated with hereditary predisposition to some form of cancer, genetic linkage analysis in affected families provides an additional route to localization. This technique depends on the crossover between homologous chromosomes at meiosis which means that DNA sequences, even on the same chromosome arm, will

not be transmitted together over several generations unless they are physically very close together. If homologous sequences on the two parental chromosomes are polymorphic, meiotic reassortment can be detected. The purpose of linkage analysis is to identify the close physical neighbors of the gene of interest in the genome (Yates and Connor, 1986). This involves finding a large number of families in which multiple individuals are affected with a specific cancer, and the testing of the individuals in these families with a large panel of DNA markers that show polymorphism. When a marker that tends to be co-inherited with the cancer gene in all (or almost all) affected members of those families is found, it indicates that the cancer gene is located near to that marker and thus allows mapping of the gene to that chromosome. Linkage analysis then proceeds by testing additional markers from the same chromosome arm and measuring the frequency of meiotic crossover between each of them and the cancer trait. In this way, the relative positions of a number of DNA sequences (including the putative cancer gene) can be established to facilitate gene isolation. The major limitation of linkage analysis in identifying tumor suppressor sequences is that it can only be used to study familial cancers and requires relatively large, well-defined pedigrees.

2.3.1.4. Molecular cytogenetics: Comparative genomic hybridization

In comparative genomic hybridization (CGH), a single hybridization allows DNA copy number changes in the entire genome of a tumor to be assessed in comparison with normal tissue DNA (Kallioniemi et al., 1992). The principle of CGH is simple. Representative genomic DNA is prepared separately from tumor and normal tissue of the same patient. The DNAs are labeled with two different fluorochromes, e.g., one with a green fluorochrome, and the other with a red. Equal amounts of these labeled DNAs are mixed and simultaneously hybridized to normal human metaphase spreads. The relative amounts of tumor and normal DNA probes bound at a given chromosomal locus

depend on the relative abundance of these sequences in the two DNA samples and can be quantitated by measurement of the ratio of green to red fluorescence along the length of each chromosome. The normal DNA probes serve as a control for local variations in the ability to hybridize to the target chromosome. Therefore, where there has been either a deletion or an amplification of DNA in the tumor, the green-to-red ration will deviate from the norm. This method has led to the localization of several susceptibility loci for human cancers (Hemminki et al., 1997; Kallioniomi, 1997).

2.3.2. Isolation of tumor suppressor genes

Once the approximate location of a putative tumor suppressor gene is mapped within two flanking markers, several methods can be used to clone the gene.

The first method is the candidate gene method. If there are one or several known genes in the region of interest, these then become "candidates" for the gene causing the cancer in question and are examined, for any mutations that segregate with the disease in affected families and for a putative function that may plausibly account for the observed phenotype. This procedure led to the identification of the *p53* tumor suppressor gene.

By using multiple probes on the short arm of chromosome 17, Baber et al. (1989) defined a region that was commonly lost in colon carcinomas. This region contains the site of the *p53* gene. The p53 protein was implicated in tumorigenesis because of its interaction with the transforming large T antigen of simian virus 40. Since no alteration in the remaining p53 locus could be detected in tumors, the p53 cDNA from tumors that had lost one p53 allele was sequenced. This analysis with two tumor samples found the presence of single point mutations occurring in evolutionarily conserved regions (Finlay et al., 1988). Additional cDNA sequencing showed that many different human tumors commonly contained mutant p53 proteins (Hollstein et al., 1994; Lane, 1994).

The second method is chromosome walking. This technique was used by Dryja et al. (1986) to clone the *Rb* tumor suppressor gene. They showed that a chromosome 13 sequence that mapped to q14 detected a region that was homozygously deleted in the tumor DNA from two unrelated Rb patients. This sequence was then used in a chromosome walking to identify a DNA fragment conserved in mouse and human, which suggested that it contained a coding sequence. This DNA fragment identified a 4.7 kb mRNA, which was expressed in normal retinal cells, but was altered or not expressed in retinoblastomas (Friend et al., 1986; Lee et al., 1987)

Another approach involves a direct search for transcribable sequences. Currently two methods are frequently used: hybridization selection and exon trapping (Duyk et al., 1990; Nehls et al., 1994; Datson et al., 1996). With hybridization selection, one first screens a genomic library with the marker sequences believed to lie close to the gene of interest. Then one screens a cDNA library from an appropriate tissue with the labeled genomic clone to isolate the cDNA. With exon trapping, genomic DNA of interest is partially digested with a restriction enzyme and cloned into a vector containing a splice donor and an acceptor sites positioned flanking the inserted genomic DNA. RNA transcripts derived from such vectors are processed in vivo and exons contained within the inserted genomic fragments become flanked by known sequences in the resulting mRNAs. Reverse transcription-coupled PCR can then be used for subsequent cloning and sequence analysis of trapped exons. Identification of cDNA by either method results in a candidate tumor suppressor gene. Confirmation that it is the tumor suppressor gene usually requires that mutations that segregate with cancer patients can be identified in that gene or that its biological function can account for the phenotype.

3. Function of tumor suppressor genes

So far, about a dozen confirmed tumor suppressor genes have been cloned (Sager,

1997). Their biological functions range from transcriptional regulation to cell cycle check point control. I will describe the functions of three tumor suppressor genes that have been extensively studied, namely *p53, Rb* and *p16*.

3.1. Function of the *p53* tumor suppressor gene

More than 50% of human tumors contain mutations in the *p53* gene (Hollstein et al., 1994). The *p53* tumor suppressor gene encodes a 393 amino acids nuclear phosphoprotein. The protein has three structural and functional domains. The N-terminal region of p53 protein is highly charged and acts as a transcription activation domain. It interacts with the basal transcription machinery to regulate gene expression (Lu and Levine, 1995). p53 transcription activation is negatively regulated by MDM2 protein which can act as an oncoprotein (Lin et al., 1995). The core or central region of p53 is highly conserved in evolution and has sequence-specific DNA binding activity. More than 90% of p53 missense mutations reside in this region. The C-terminal domain of p53 protein is responsible for its oligomerization. p53 proteins assemble through this domain to form stable tetramers. The last 28 amino acids of p53 act as a site of negative regulation for the sequence-specific DNA binding function of the central domain (Hupp and Lane, 1994) and may also encode a non-specific DNA binding site (Levine, 1997).

Normally the amount of p53 protein in a cell at steady state is low because of its relative short half life (about 20 minutes). Different types of DNA damage can activate p53 protein, including double-strand breaks induced by γ-irradiation, ultraviolet irradiation, and chemical damage to DNA. This results in a rapid increase in the level of p53 protein in cells and activation of p53 as a transcription factor (Cox and Lane, 1995). One of the downstream genes of p53 is the p21 protein, an universal inhibitor of cyclin-dependent kinases (El-Deiry et al., 1993). The p21 protein binds to a number of cyclin-

cdk complexes, inhibiting cdk kinase activity and blocking cell cycle progression (Xiong et al., 1993). The p21 protein also binds to proliferating cell nuclear antigen (PCNA), blocking its role as a DNA polymerase processitivity factor in DNA replication (Waga et al., 1994). Therefore, p21 can act on cyclin-cdk complexes and PCNA to stop DNA replication, and arrest cells in the G1 phase. This p53-mediated G1 arrest is believed to give cells sufficient time to repair DNA damages before the cells enter into the S phase.

The p53 protein has also been shown to mediate G2/M phase arrest. When mitotic spindle inhibitors, such as nocodazole, are added to cells with wild type p53, the cells are arrested in G2. In the absence of p53, these cells will reinitiate DNA synthesis, increasing the ploidy of the cells (Cross et al., 1995). In addition, p53 appears to be involved in regulating the number of centrosomes in a cell. Mouse embryo fibroblasts (MEFs) from p53 null mice produce abnormal numbers of centrosomes after a few doublings in cell culture and initiate spindles with three or four poles, whereas MEFs from normal mice in culture at the same passage level do not exhibit this phenotype (Fukasawa et al., 1996). This p53-mediated G2/M check point may account for the phenotype of genomic instability that is commonly associated with a p53 mutation.

Another important function of the p53 protein is to regulate apoptosis. Normal thymocytes will undergo apoptosis in response to DNA damage, whereas thymocytes from p53 null mice do not (Lowe et al., 1993). p53 can also initiate apoptosis in response to the expression of a viral or cellular oncogene. The expression of the adenovirus E1A protein in rat fibroblasts stabilizes and activates p53 protein. The resultant cells die of apoptosis (Debbas and White, 1993). Cells overexpressing E2F-1 and a temperature sensitive mutant p53 protein undergo apoptosis at 32^0C but not at 37-39^0C, where p53 is inactive (Wu and Levine, 1994). Similarly, cells overexpressing myc and a temperature-sensitive p53 have a temperature-sensitive apoptotic response

(Wagner et al., 1994).

Besides these factors, hypoxia is able to stimulate p53 levels and induce apoptosis (Graeber et al., 1996). It has been suggested that this process represents another way that p53 may act as a tumor suppressor (Kinzler and Vogelstein, 1996). Most tumors are initiated by genes other than p53 and go through many rounds of clonal expansion to reach a critical size when the blood supply becomes rate-limiting. Angiogenesis is required for clinically apparent growth of a tumor. The resultant hypoxia induces a p53-mediated apoptosis to limit the progression of tumors.

In summary, the current knowledge of p53 function suggests that the p53 protein acts to protect the organism from genetic damage. In response to damage or potential damage to the DNA, p53 protein initiates a protective cell cycle arrest or apoptotic cell death. A number of factors affect the decision of a cell to enter a p53-mediated cell cycle arrest or apoptotic pathway. Under conditions in which the DNA is damaged, growth factors are deprived, or an activated oncogene is forcing the cell into proliferation, p53-mediated apoptosis prevails. In this way, cells with damaged DNA or cells in a stressed environment are eliminated in a p53-dependent apoptotic event. Loss of p53 gene function allows the propagation of cells with genetic damage and this may be a key step in the development of neoplasia (Lane, 1994; Levine, 1997).

3.2. Function of the Rb tumor suppressor gene

Rb, the product of the retinoblastoma tumor suppressor gene, exerts its cell growth inhibitory function mainly by inhibiting the activity of transcription factors E2Fs. E2Fs are a family of heterodimeric transcription factors that can transactivate genes whose products are important for S phase entry, including most notably c-myc, B-myb, cdc2, thymidine kinase and dihydrofolate reductase (Nevins, 1992; Lathangue, 1994).

Rb exerts most of its effect in a defined window of time in the first two thirds of the G1

phase of the cell cycle. Cells entering G1 from mitosis require exposure to serum mitogens continuously until several hours before the onset of S phase; thereafter they become relatively serum-independent. This transition from a serum-dependent to serum-independent state is demarcated by a discrete point in time, termed R (restriction) point (Pardee, 1989). Before a cell reaches the R point, the Rb protein is found in an underphosphorylated form. The hypophosphorylated Rb binds to a subset of E2F complexes, converting them to repressors that constrain expression of E2F target genes (Weinberg, 1995; Sherr, 1997). During the last several hours of G1, the Rb protein is initially phosphorylated by cyclin D-dependent kinases and then by cyclin E-CDK2 complex. Phosphorylated Rb protein dissociates from E2Fs, enabling them to transactivate the genes important for S phase entry.

A diverse body of evidence indicates that the Rb protein is involved in the pathogenesis of a variety of human tumors. In retinoblastomas, in small cell lung carcinomas, and in many sarcomas and bladder carcinomas, Rb function is lost through mutations of the *Rb* gene (Horowitz et al., 1990). In the great majority of cervical carcinomas, the cells have been previously infected with one of the oncogenic forms of the papilloma virus and the inactivation of Rb is achieved by the binding of the E7 viral protein (zur Hausen, 1991). As discussed earlier, many tumor cells constitutively overexpress cyclin D1 (see B.3.6), and this in turn activates cyclin D-dependent kinases and phosphorylates Rb protein. Taken together, as a direct consequence of Rb inactivation (either by genetic mutation or by functional inactivation), E2Fs are liberated from Rb inhibition and the progression of cells into late G1 and S phase become uncontrolled.

3.3. Function of the p16 tumor suppressor gene

The cell cycle is controlled by the sequential activation and inactivation of cyclin-

dependent kinases. As discussed earlier, among the members of the cyclin family, D1 can be an oncogene and functions as an effector of mitogen-induced proliferation acting during the G1 phase of the cell cycle. The D1-dependent kinases are subject to additional levels of regulation, including the association with inhibitory subunits (Morgan, 1995; Sherr and Roberts, 1995). One such inhibitor, p16, specifically binds and inhibits the cyclin D-dependent kinases by competing with D cyclins. Since cyclin D-dependent kinase activity is required to phosphorylate the Rb protein during the middle to late G1 phase as described above, p16 can negatively regulate cell proliferation by suppressing hyperphosphorylation and functional inactivation of the Rb protein.

The involvement of p16 in the development of human cancers was implied by the observation that the *p16* gene is mutated in many tumor-derived cell lines and maps to chromosome 9p21, a region frequently altered in human malignancies (Kamb et al., 1994; Nobori et al., 1994; Okamoto et al., 1994). Several mechanisms of p16 inactivation have been characterized. Point mutation and small deletions are common in pancreatic adenocarcinomas, esophageal carcinomas, and in families with hereditary susceptibility to melanoma (Hirama and Koeffler, 1995; Pollock et al., 1996). Homozygous deletions of the p16 locus occur commonly in non-small cell lung carcinomas, head and neck tumors, prostate tumors and bladder carcinomas (Hirama and Koeffler, 1995). Finally, methylation of the *p16* gene locus is common in breast and colon cancers and results in silencing of the p16 promoter (Herman et al., 1995). Consistent with its tumor suppressor function, p16 null mice spontaneously develop a spectrum of different tumors by 6 months of age, with the rate of tumor formation accelerating in response to carcinogen treatment (Serrano et al., 1996). Cultured $p16^{-/-}$ embryo fibroblasts do not enter senescence, and unlike their wild type counterparts, they can be transformed by oncogenic ras alone (Serrano et al., 1996). Presumably,

p16 loss might mimic the overexpression of cyclin D1, leading to the hyperphosphorylation and inactivation of Rb protein.

D. Carcinogenesis is a multistep process: The role of oncogene and tumor suppressor gene

1. Epidemiological studies of cancer

Cancer is predominantly a disease of the elderly, with the risk of acquiring this disease increasing with age. The first clue to the multistep nature of carcinogenesis comes from epidemiological studies. Earlier epidemiological studies of cancer incidence as a function of age using mathematical modeling have shown that for adult human tumors four to six genetic changes were required for genesis of a tumor (Armitage and Doll, 1954; Peto, 1977; Dix, 1989). Recently Renan (1993) used the same method but a better defined data set to address the question of the number of mutational changes required for 28 different human malignancies. By plotting the log of the age-specific mortality rate against the log of the age in years of the person affected, and determining the best-fit linear regression coefficients for each tumor type, he concluded that the common adult human cancers of the lip, stomach, liver, pancreas, kidney, skin and bladder required 7 to 8 mutational changes, and tumors with very late onset, such as prostate cancer, required 12 changes. Though this type of studies provides us a general idea about the multistep nature, it can not tell what genetic changes are involved.

2. Experimental animal studies: Mouse skin carcinogenesis

A classical model of multistep carcinogenesis is the pathogenesis of mouse skin cancer (Mottram, 1944; Boutwell, 1964; 1974; Hennings et al., 1990; Yuspa, 1994). Sequential application of chemical agents to mouse skin can induce tumors, and tumor development can be divided into three stages: initiation, promotion and progression. Typically, tumor initiation is brought about by the single application of a mutagen, such

7,12-dimethylbenz(a)anthracene (DMBA); promotion is carried out by repeated application of a phorbol ester, such as 12-O-tetradecanoylphorbol-13-acetate (TPA) or by a natural promoting stimulus such as wounding. Papillomas begin to appear at 12 to 20 weeks after the promotion has begun and by about 1 year, about 40% to 60% of the animals have some papillomas that become squamous cell carcinomas. If the promoting agents are given alone or before the initiating agent, usually no malignant tumors occur.

Detailed studies of the molecular basis of each stage of the skin tumor development indicate that initiation involves a permanent, heritable changes in the gene expression of the initiated cells which produces a subtle change in the keratinocyte phenotype. Genetic analyses revealed that c-Ha-ras alterations are associated with the initiated phenotype. It has been shown that c-Ha-ras mutations are usually heterozygous in papillomas and can be detected in initiated skin prior to the emergence of tumors (Nelson et al., 1992). Furthermore, the initiating agent used determines the existence, nature, and site of the c-Ha-ras mutation (Quintane et al., 1986; Brown et al., 1990). For example, when DMBA is metabolized, the diol epoxide produced primarily binds to adenine residues in DNA (Cheng et al., 1988). When H-ras mutations were analyzed in skin tumors of DMBA-treated mice, the mutations were A_{181} to T transversions in 45 of 50 tumors (Brown et al., 1990).

When a subpopulation of keratinocytes isolated from carcinogen-initiated skin are cultured in vitro, they resist the Ca^{2+} signal for terminal differentiation and evolve as foci which continue to grow in medium containing >0.1 mM Ca^{2+}. Biochemical analyses indicate that overexpression of the TGFα protein and an alteration of PKC are essential for mediating this phenotype. Differential modification of PKC isoforms, particularly activation of PKCα through increased levels of cellular diacylglycerol and functional

inhibition of PKCδ by tyrosine kinase, produces keratinocytes with enhanced proliferative capacity and reduced sensitivity to signals for terminal differentiation (Yuspa, 1994).

Application of tumor promoters to initiated epidermis causes the selective clonal outgrowth of initiated cells to produce multiple benign squamous cell papillomas, each representing an expanded clone of initiated cells (Deamant et al., 1987; Iannaccone et al., 1987). The mechanisms of exogenous promotions are likely to be epigenetic in most cases because (1) a single genetic change in normal keratinocytes is sufficient to produce a papilloma phenotype (Greenhalgh et al., 1993) and (2) most promoting agents are not mutagens (Yuspa and Dlugosz, 1991).

Progression of a papilloma to a carcinoma in mouse skin is usually a spontaneous process. Progression and malignant conversion can be enhanced and accelerated by exposing animals bearing papillomas to a mutagen, supporting a genetic basis for progression (O'Connell et al., 1986; Hennings et al., 1990). Genetic studies indicate that non-random, sequential chromosomal aberrations are associated with progression of mouse skin papillomas; particularly prominent are trisomies of chromosomes 6 and 7 (Yuspa, 1994). Changes in two cellular genes, c-Ha-*ras* and *p53*, have been closely identified with malignant conversion of skin tumors. The mutated c-Ha-*ras* gene, which is heterozygous in papillomas, is frequently homozygous in carcinomas (Bianchi et al., 1990). Mutations in the *p53* tumor suppressor gene are rarely found in chemically induced papillomas but are frequently detected in squamous carcinomas, particularly those induced by benzo(a)pyrene (Ruggeri et al., 1991; 1993; Kress et al., 1992). Recent studies indicate that 90% of squamous carcinomas are devoid of TGFβ1 and TGFβ2. Direct evidence linking TGFβ1 loss and accelerated malignant tumor progression comes from studies using keratinocytes cultured from TGFβ1 null mice.

Introduction of v-Ha-*ras* oncogene into cultured keratinocytes of TGFβ1 null mice or wild-type and heterozygous littermates results in papillomas when these cells are grafted to nude mice. However, carcinomas only develop in the TGFβ1 null papillomas but not in those of other genotypes (Yuspa, 1994). These results indicate that the TGFβ family of growth inhibitors can serve as suppressor of malignant progression.

The mouse skin carcinogenesis model clearly demonstrates that the genesis of skin tumors requires non-genetic changes as well as a series of genetic changes. Mutations in oncogenes, e.g., c-Ha-*ras*, tumor suppressor genes, e.g., *p53*, altered expression of TGFβ1, altered activity of PKC and characteristic chromosomal abnormalities all contribute to the development of the squamous cell carcinomas.

3. Human cancer studies: Colorectal carcinogenesis

Colorectal carcinomas have proven to be an excellent model system in which to study the genetic changes involved in the initiation and progression of human solid tumors. There is a well-defined progression from benign to malignant tumors, and the cancers that arise are clonal in origin. Tumors of various stages of dysplasia and malignancy, ranging from benign adenomas to invasive carcinomas, can be obtained surgically. In addition, colorectal cancer exists in both sporadic and inherited form. For example, in patients with familial adenoma polyposis (FAP), hundreds to thousands of adenomatous polyps will develop in their colons and rectums, and a small percentage of these go on to become malignant. The high frequency of polyps at various stages of progression allows one to study the stage-wise pattern of colon cancer. Studies of this inherited cancer syndrome and large number of sporadic tumors allowed Vogelstein and colleagues to identify the critical genetic events driving colorectal carcinogenesis (Vogelstein et al., 1988; Fearon and Vogelstein, 1990).

An early change that occurs in the genome of cells of small, early adenomas from FAP

patients and patients without familial predisposition is chromosomal allelic loss of 5q. Linkage analysis and molecular cloning have identified the affected gene on chromosome 5q21 in FAP and the gene is called *APC* (for adenomatous polyposis coli) (Groden et al., 1991; Kinzler et al., 1991). The *APC* gene is mutated in the germline of FAP patients (Mandl et al., 1994) while truncating APC mutations have been identified in 60% of sporadic colorectal cancers and adenomas (Powell et al., 1992; Ichii et al., 1993). Both copies of APC are inactivated either by mutations on each allele, or by a mutation on one allele and a structural deletion in the other. The data indicate that *APC* is a tumor suppressor gene, and one or both alleles of the *APC* gene are lost orinactivated at an early stage in the development of colorectal cancer.

Mutations in the K-*ras* oncogene have been identified in about 10% of small adenomas, 50% of larger adenomas and in 50% of carcinomas. Such ras mutations, usually in codons 12 or 13, tend to be observed in more dysplastic adenomas. *Ras* gene mutations appear to occur in one cell of a preexisting adenoma and confers it a growth advantage. Through clonal expansion of the cells with the mutation, a small adenoma is converted into a larger and more dysplastic one.

Loss of heterozygosity of additional tumor suppressor genes appears to be critical in later stages of colorectal tumorigenesis. The chromosomes most frequently deleted include chromosome 18q and 17p. 18q is deleted in 50% of late adenomas and more than 70% of carcinomas (Vogelstein et al., 1988). A candidate tumor suppressor gene from this region has been identified. This gene, termed *DCC* (for deleted in colorectal cancer), encodes a protein with putative cell adhesion properties. Its expression is absent or reduced in colorectal carcinomas, suggesting that its involvement in normal cell-cell or cell-matrix interactions is required to maintain a normal state of differentiation. The loss of a large portion of one copy of chromosome 17p has been

observed in more than 75% of carcinomas. This loss is rarely seen in adenomas at any stage. The common region lost on 17p contains the *p53* tumor suppressor gene. Nucleotide sequencing analysis of the p53 cDNA derived from colorectal cancers has shown that in 70% to 80% of the cases there is a missense mutation in the remaining p53 allele in the cancer cells. Additional chromosome losses, including 1q, 4p, 6q, 8p, 9q and 22q, has been observed in colorectal cancers. On average, colorectal carcinomas contain four or five allelic losses. Patients with more than the median number of losses in their tumors have a poorer prognosis.

In summary, colorectal tumors appear to arise as a result of the mutational activation of specific oncogenes and inactivation of specific tumor suppressor genes. Mutations in at least four to five genes are required for the formation of a malignant tumor. Fewer changes are sufficient for benign tumorigenesis. These studies clearly demonstrate the complex multistep nature of human tumor development.

4. Transformation of human cells in culture

One of the advantages in using human cells in culture to study neoplastic transformation is that it provides a means to dissect the carcinogenesis process under well-defined conditions. However, normal human cells in culture have never been found to undergo spontaneous malignant transformation and have proven to be extremely difficult to be malignantly transformed by any means (McCormick and Maher, 1988; Rhim, 1993). Recently neoplastic transformation of human cells in culture has been achieved in a stepwise fashion-immortalization and malignant conversion of the immortalized cells. These studies support a multistep process for neoplastic transformation and provide insights into the molecular mechanisms underlying the process.

4.1. Immortalization of human cells in culture

Normal human cells in culture have a limited life span, beyond which the cells enter the terminally nondividing state referred to as senescence (Hayflick and Moorhead, 1961). Finite life span normal human cells can only undergo two successive clonal selections before they enter crisis and senesce (McCormick and Maher, 1988). Earlier studies trying to transform finite life-span human cells in culture were not successful, suggesting that acquiring immortality is a prerequisite if a cell is to acquire sequentially all the genetic changes needed to become malignant (Kuroki and Huh, 1993; McCormick and Maher, 1994). Whether this is the case for cells in the human body is not known for certain. What is known is that many malignant tumor-derived cells can be grown indefinitely in culture, whereas cells from normal tissues are never able to grow indefinitely in culture.

Human cells can be immortalized by repeated treatment with chemical and physical carcinogens, and by infection or transfection with certain viral genes. However it occurs at a very low frequency (McCormick and Maher, 1988; Shay et al., 1991; Bai et al., 1993; Kuroki and Huh, 1993). In order to immortalize human fibroblasts, for example, Namba et al. (1988) had to expose them to more than 10 treatments with either 4-nitroquinoline 1-oxide or gamma-irradiation and has been successful only a few times. Nevertheless, immortalization of human epithelial cells, for example, epidermal keratinocytes, bronchial epithelial cells, mammary epithelial cells, and prostate epithelial cells, have been achieved by infection with the AD12-SV40 hybrid virus or human papilloma virus (Kuroki and Huh, 1993; Rhim, 1993).

Immortalization of diploid human foreskin fibroblast has been achieved in this laboratory by transfection with a v-myc oncogene. An early passage, foreskin-derived normal human fibroblast cell line was transfected with a plasmid carrying the neo gene and a v-myc gene. The transfectants were selected for geneticin resistance and clonally-derived

cell strains expressing the v-myc protein were isolated and propagated for many generations. Eventually all cell strains senesced, but among the senescing progeny viable cells could be seen. These eventually gave rise to an infinite life span cell strain designated MSU-1.1 (Morgan et al., 1991). This experiment demonstrates that expression of the v-myc oncoprotein alone is not sufficient to cause human fibroblasts to acquire an infinite life span, but we do not yet know what additional genetic change(s) is required.

At present the mechanisms underlying immortalization are poorly understood. Careful analyses of the immortalization process suggest that immortalization is caused by multiple genetic changes. For example, following infection by SV40 virus or stable transfection with the SV40 T-antigen, normal proliferating human fibroblasts replicate for about 20-30 more population doublings beyond their normal senescent point. The population then enters a state of crisis, during which cell number remains constant or declines due to an increase in cell death. From this crisis population arise the rare, immortal clones, presumably due to additional genetic events (Wright and Shay, 1992). It has been reported that (Shay and Wright, 1989) for a population of SV40 T-antigen transfected human lung fibroblasts, the frequency of immortalization is about 3×10^{-7}. Somatic cell hybridization between an immortal cell line and a finite life span cell line results in cells with limited life span, indicating that immortalization is caused by recessive gene mutations (Smith and Pereira-Smith, 1996). Recently, a number of studies indicate that the *p53* and *Rb* tumor suppressor genes may play a causal role in immortalization of certain cell types (Shay et al., 1991; Vojta and Barrett, 1995). For example, fibroblasts from seven out of eight Li-Fraumeni syndrome patients escaped senescence and were immortalized spontaneously in culture (Bischoff et al., 1990). Hara et al. (1991) reported that targeted functional knock-out of the *Rb* and *p53* with

antisense oligomers resulted in an extended life span of human fibroblasts.

4.2. Neoplastic transformation of immortal human cells

While immortality is not sufficient for transformation, most immortalized cells have an increased sensitivity for spontaneous, carcinogen- or oncogene-induced neoplastic progression. For example, AD12-SV40 hybrid virus immortalized human epidermal keratinocytes can be malignantly transformed by retroviral oncogenes such as H-*ras*, *fms*, *erbB* and *src*; they can also be transformed by chemical carcinogens or by x-ray irradiation treatment (Reviewed by Rhim, 1993). This laboratory has successfully converted MSU-1.1 human fibroblasts into malignant cells by transfection of an activated H-*ras* or an N-*ras* oncogene in a vector engineered for overexpression of the oncogene. Expression of the same *ras* oncogenes at the level found for the endogenous H-*ras* or N-*ras* proto-oncogene did not cause malignant transformation. However, if MSU-1.1 cells that expressed a transfected H-*ras* or v-*sis* oncogene at low levels were subsequently transformed with a v-*fes* oncogene, then the cells became malignant (Lin et al., 1995). These studies indicate that more than one genetic changes are needed for the malignant transformation of MSU-1.1 cells. They also demonstrate the complementary role between oncogenes.

In summary, normal human cells in culture can be immortalized by a variety of means (viruses, chemical carcinogens, irradiation and oncogene transfection). Additional exposure of these immortal cells to a carcinogenic agent and appropriate selection can result in malignantly transformed cells. Thus these studies demonstrate that similar to tumor development in vivo, neoplastic transformation of human cells in culture is indeed a multistep process.

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CHAPTER II

Suppression of Anchorage-independent Growth and Matrigel Invasion and Delayed Tumor Formation by Elevated Expression of Fibulin-1D in Human Fibrosarcoma-derived Cell Lines

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ABSTRACT

Using differential display, we identified an mRNA that is markedly down-regulated in cell line 6A/SB1, derived from a fibrosarcoma formed in an athymic mouse following injection of carcinogen-transformed MSU-1.1 cells. The nontumorigenic parental cell strain, MSU-1.1, expresses high levels of this mRNA. Sequencing of the corresponding cDNA fragment revealed that it corresponded to an expressed sequence tag, which ultimately led to its identification as the *fibulin-1D* gene. Fibulin-1 is a cysteine-rich, calcium-binding extracellular matrix and plasma protein, which has four isoforms A-D derived from alternative splicing. Northern and Western blotting analysis of 16 cell lines established from tumors formed in athymic mice by MSU-1.1-derived cell strains independently transformed in culture showed that 44% exhibited low level or lack of expression of fibulin-1D mRNA and protein. In a similar analysis of 15 malignant cell lines derived from patients, 80% showed low level or no expression. To study the role of fibulin-1D in transformation, we transfected 6A/SB1 cells and a human fibrosarcoma-derived cell line (SHAC) with a fibulin-1D cDNA expression construct. Transfectants displaying high levels of fibulin-1D were isolated and characterized. Elevated expression of fibulin-1D led to reduced ability to form colonies in soft agar and reduced invasive potential as tested in a matrigel in vitro invasion assay. Furthermore, expression of fibulin-1D resulted in a markedly extended latency in tumor formation in athymic mice. These results indicate that low expression of fibulin-1D plays a role in tumor formation and invasion.

INTRODUCTION

Extensive research over the past two decades has established that cancer in humans arises from a cell that has acquired multiple genetic alterations in oncogenes and/or tumor suppressor genes (Weinberg, 1989; Fearon and Vogelstein, 1990; Hunter, 1991). Mutations and/or altered expression of these critical genes and their downstream effector genes allow a cell to escape from normal growth control and become malignant. A direct comparison of gene expression between non-tumorigenic and tumorigenic cells of the same genetic background should provide information on genetic changes involved in the transformation process. Subsequent cloning and functional analysis of these differentially expressed genes may enrich our understanding of cancer development.

To study the molecular mechanisms underlying neoplastic transformation of human cells, we used the near-diploid, karyotypically stable, infinite life span human fibroblast cell strain MSU-1.1 as a model system. Cell strain MSU-1.1 was established in this laboratory from a normal diploid human neonatal foreskin fibroblast cell line (Morgan et al., 1991). The cells are phenotypically normal and do not form tumors in athymic mice. Transfection of MSU-1.1 cells with an activated *ras* oncogene expressed at high levels (Hurlin et al., 1989; Wilson et al., 1990) or treatment of the cells with chemical carcinogens, such as (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) followed by selection for focus-forming cells (Yang et al., 1992) results in transformants capable of tumor formation in athymic mice. However, the molecular basis of the neoplastic conversion in MSU-1.1 cells by treatment with chemical carcinogens is not understood. To identify putative oncogene(s) and tumor suppressor gene(s), as well as other types of genes associated with malignant transformation, we used differential mRNA display (Liang and Pardee,

1992; Liang et al., 1993) to compare the profile of gene expression in parental cell strain MSU-1.1 and cell line 6A/SB1. The latter was derived from a fibrosarcoma formed in an athymic mouse by a BPDE-transformed MSU-1.1 cell strain. One of the differentially expressed genes identified corresponds to fibulin-1D, an extracellular matrix (ECM) and plasma protein (Argraves et al., 1990, Tran et al., 1997). It is expressed at high levels in MSU-1.1 cells and is markedly down-regulated in 6A/SB1 cells. Analysis of 16 cell lines established from tumors formed in athymic mice by MSU-1.1-derived cell strains transformed in culture by various agents showed that seven (44%) had a low level or complete lack of expression of fibulin-1D mRNA and protein. Similarly, analysis of 15 cell lines derived from tumors taken from patients showed that 12 (80%) had a low or complete lack of expression of this mRNA and protein.

To determine if low expression of fibulin-1D were causally involved in the malignant transformation of these cell lines, we studied the effect of stably expressing fibulin-1D in human fibrosarcoma-derived cell line SHAC and in 6A/SB1cells, which lack the expression of this protein. The results showed that elevated expression of fibulin-1D significantly decreased the anchorage-independent growth of both SHAC and 6A/SB1 cell derivatives, as well as their invasiveness when tested in a matrigel in vitro invasion assay, and significantly delayed the onset of tumor formation in athymic mice by these cell lines.

RESULTS

Identification of fibulin-1D as a differentially expressed mRNA

To identify transformation-related genes, we carried out differential mRNA display analysis, comparing the non-tumorigenic parental MSU-1.1 cell strain and a tumorigenic derivative cell line, 6A/SB1. RT-PCR reactions were carried out using 80 different combinations of primer sets, composed of four degenerate anchored oligo(dT) primers, $T_{12}MN$ (M was dG,dC or dA; N was dG, dC, dA or dT) and 20 arbitrary 10-mers (OPA 1-20, Operon Technologies, Alameda, CA). About 8,000 bands were observed. The band pattern for cell line 6A/SB1 differed from that of parental MSU-1.1 in nine bands, each of which was down-regulated in 6A/SB1 cells. These cDNAs were excised from the sequencing gel, reamplified by PCR, and used as probes for Northern blot analysis. Such analysis showed that only five of these nine differentially-displayed cDNAs represented mRNAs that actually are expressed at significantly lower levels in the 6A/SB1 cells as compared with the MSU-1.1 cells. One of the five unique bands, designated SG7, is characterized in this report. As shown in Figure 1B, this cDNA hybridized to a 2.7-kb transcript showing a 14-fold lower level of expression in 6A/SB1 cells than in MSU-1.1 cells. Cloning and sequencing of this partial cDNA indicated that it consisted of 311 base pairs and was 98.7% identical to an expressed sequence tag (Genbank accession No. T19384).

To get the full-length cDNA corresponding to SG7, EST clone T19384 was obtained from the investigator who reported it, Dr. Georges Guellaen (Hopital Henri Mondor, Creteil, France), and the 5' and the 3' regions of the EST clone were sequenced. A database search revealed that the EST corresponded to the human *fibulin-1D* gene, with 97.4% identity in a 312-bp overlap in the 5' region (bases 1-312 of fibulin-1D) and 98.9% identity in a 188-bp overlap in the 3' region (bases 2172-2359 of fibulin-1D). The

SG7 cDNA that we isolated by differential display contained a sequence that extended beyond the 3' sequence of the fibulin-1D cDNA sequence deposited in the database.

To confirm the identity of SG7 as human fibulin-1D, we carried out Northern blot analysis using the 2.3-kb human fibulin-1D cDNA insert from plasmid pBluescriptSKfibulin1D (Tran et al., 1997) to probe the same membrane previously used to detect SG7 expression. As shown in Figure 1C, the fibulin-1D cDNA probe detected the 2.7-kb mRNA transcript that had been detected by SG7 (Fig.1B), but it also detected a 2.3-kb mRNA transcript which corresponds to fibulin-1C. This is consistent with the fact that these two mRNAs have identical 5' regions (bases 1-1707). Both transcripts exhibited coordinately decreased expression in 6A/SB1 cells compared with the parental MSU-1.1 cells.

Expression analysis of fibulin-1D in multiple human tumor cell lines

To test whether the decreased expression of fibulin-1D transcript is a common feature of human tumor cells, we carried out Northern analysis using fibulin-1D cDNA as a probe with RNA from 15 cell lines derived from malignant tumors taken from patients and 16 cell lines established from tumors produced in athymic mice by MSU-1.1-derived cell strains transformed in culture by oncogene transfection or carcinogen treatment. The origin of these cell lines is listed in Table 1. Examples of results are shown in Figure 2. Very low levels of expression of fibulin-1 were found in five out of five fibrosarcoma-derived cell lines (lanes 15-19), in two out of two osteosarcoma-derived cells (lanes 8 and 9), in two out of three neurofibrosarcoma-derived cells (lanes 12 and 20), in one out of two human bladder carcinoma-derived cells (lane 11), and in the rhabdomyosarcoma- and cervical carcinoma-derived cell lines (lanes 7 and 10) tested in this study. Downregulation of fibulin-1 mRNA expression was also found in 6 out of 16 MSU-1.1 lineage-derived tumor cells (lanes 3-6, and 22-23), in addition to cell strain

6A/SB!, in which the downregulation was first identified (Fig. 1). Taken together, 19 out of 31 tumor-derived cell lines exhibited low or no expression of fibulin-1 (C and D transcripts).

To determine whether mRNA levels correlate with fibulin-1 protein expression, we performed Western blot analysis of cell extracts and their serum-free conditioned culture media. As a control, purified fibulin-1 protein from human placenta was included in each assay. As shown in Figure 3, lane 1, under non-reducing conditions, mouse-anti-human fibulin-1 monoclonal antibody 3A11 specifically recognized the fibulin-1 protein (apparent molecular weight, 80 kDa). We found that, consistent with results from Northern analysis, the expression of fibulin-1 protein was very low or undetectable in a panel of tumor cell lines, not only in the cell extracts (Fig.3A) but also in their conditioned culture media (Fig.3B), whereas a neonatal foreskin-derived human fibroblast line, LG1, and MSU-1.1 cells expressed high levels of fibulin-1 proteins. The presence of multiple immunoreactive polypeptides that migrated faster than the mature fibulin-1 polypeptide on SDS-PAGE (Fig.3A) may correspond to proteolytic fragments of fibulin-1. Similar fragments have been observed in purified fibulin-1 preparations derived from placenta and lung and have been generated in vitro by matrilysin and leucocyte elastase digestion (Sasaki et al, 1996).

Establishment of SHAC and 6A/SB1 clones stably expressing high levels of fibulin-1D protein

The levels of fibulin-1 protein in either cell extracts or conditioned media (Fig.3) from fibrosarcoma-derived cell lines SHAC and 6A/SB1 is almost undetectable. To examine the effect of fibulin-1D expression on malignant transformation, we constructed a human fibulin-1D cDNA expression vector, designated pPuro-Fibulin-1D, and transfected it into SHAC and 6A/SB1 cells. Puromycin resistant clones were isolated from cells that were

Fig.1. (A) Differential display comparing mRNAs from the parental MSU-1.1 cell strain (lanes 1 and 2) and its malignant derivative cell line 6A/SB1 (lane 3 and 4). For each cell line, two independent preparations of total RNA were extracted, reverse transcribed, and amplified by PCR in the presence of [α-^{35}S]dATP. The PCR products were separated on a 6% polyacrylamide gel and autoradiographed. The signal demonstrating altered expression is marked by an arrow. Primers used were T_{12}MG and OPA7. (B) Northern blot analysis confirming differential gene expression for fragment SG7 using the cloned cDNA fragment as a probe. Total RNA (15 μg) obtained from MSU-1.1 cells (lane 1) and 6A/SB1 cells (lane 2) was subjected to Northern analysis as detailed in "Materials and Methods". The blot was stripped and reprobed with a GAPDH cDNA (lower panel) as the loading control. (C) Northern blot analysis using fibulin-1D cDNA as a probe. The same blot of (B) was stripped and hybridized with a radiolabeled 2.3-kb human fibulin-1D cDNA insert from the pBluescriptSKfibulin-1D plasmid.

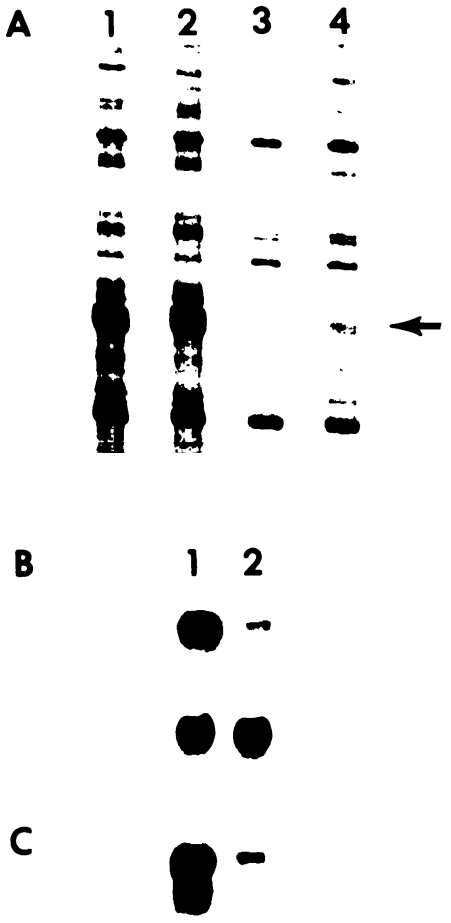

Fig.1

the result of independent transfection events, i.e., from independent dishes. Immunoblot analysis of whole cell extracts was used to identify clones that expressed high levels of fibulin-1D protein. For SHAC cells, a total of 24 clonally-derived, drug-resistant populations were tested. Five independent clones exhibited high levels of fibulin-1D protein. Two clones of SHAC cells that had been transfected with the control vector pBABE-Puro and displayed no detectable fibulin-1D protein were used as negative controls. The same procedure was carried out with the 6A/SB1 cells. Of 45 puromycin-resistant clones assayed, 10 showed high expression of fibulin-1D. Six independent positive clones and three vector-transfected negative control clones were selected at random for further characterization.

We also evaluated the level of fibulin-1 secreted into the conditioned culture medium by these cell strains. As expected, neither the parental cells nor the vector-transfected control clones secreted fibulin-1. In contrast, the transfected clones that expressed high levels of cellular fibulin-1D secreted relatively high levels of mature proteins into the medium (Fig.4).

Anchorage-independent growth of the clones that express fibulin-1D

Since most malignantly transformed cells, including SHAC and 6A/SB1 cells exhibit anchorage-independent growth, i.e., do not require attachment to a substratum for proliferation, whereas normal human fibroblasts and MSU-1.1 cells do, we tested whether fibulin-1D expression affects the ability of SHAC and 6A/SB1 cells to form colonies in agarose. As shown in Figure 5 and Table 2, all the transfectant clones expressing high levels of fibulin-1D exhibited a reduced ability to grow in agarose compared to their parental cells and their vector-transfected control cell strains. The SHAC cells were counted after 21 days; the 6A/SB1 cells after 14 days. A substantial decrease in the size of colonies was observed for SHAC cell transfectants. A moderate

Table 1 Human tumor-derived cell lines tested for fibulin-1 expression

Cell lines[a]	Origin of the cell lines
L45I/B5T, L46I/7T, L55I/3T, 2FT/T1	Derived from spontaneously transformed MSU-1.1cells
2C1/ST1, 6A/SB1, 11C/SB1	Derived from BPDE-transformed MSU-1.1 cells
MSU-1.1-γ1- 2A1/T, MSU-1.1- γ4-2A/SF1[b]	Derived from ^{60}Co-transformed MSU-1.1 cells
MSU-1.2-γ1/SF1, MSU-1.2-γ2/SF1	Derived from ^{60}Co-transformed MSU-1.2 cells
2MT, 3MT, 178-2DT, DW5T, 1.1-sisB/ST2	Derived from oncogene-transformed MSU-1.1 cells
WSU1/T1, WSU17/T1	Derived from human neurofibrosarcomas
NCI, VIP:FT, SHAC, HT1080, 8387[c]	Derived from human fibrosarcomas
143 BTK, TE85	Derived from human osteosarcomas
RD	Derived from a human rhabdomyosarcoma
Hela	Derived from a human cervical carcinoma
GM03808[d]	Derived from a Wilms' tumor
T24, A1698[e]	Derived from human bladder carcinomas
NF1.90.8/FT1[f]	Derived from a human neurofibrosarcoma

[a] Cell lines in the first five lines of the table were established in this laboratory from sarcomas generated in athymic mice by injection of transformed MSU-1.1 cells; those on line six were established in this laboratory from human neurofibrosarcomas. The rest, with the exception of 8387, GM03803, A1698, and NF1.90.8/FT1, were obtained from American Type Culture Collection, Rockville, MD.

[b] Referred to as MSU-1.1-γ1 in Figure 2.

[c] From Dr. Stuart A. Aaronson, National Cancer Institute, Bethesda, MD.

[d] From Coriell Institute for Medical Research, Camden, NJ.

[e] From Dr. O. M. Pereira-Smith, Baylor College of Medicine, Houston, TX.

[f] From Dr. Thomas Glover, University of Michigan, Ann Arbor, MI.

Fig. 2. Northern blot analysis of fibulin-1 expression in multiple human tumor cell lines. Total RNA (15 μg), obtained from a series of cell lines, was hybridized with fibulin-1D cDNA as in Figure 1C. These were: fibroblast cell lines from normal donors (lanes 1 and 2); malignant cell lines derived from tumors formed in athymic mice after injection of MSU-1.1 cells transformed by a transfected oncogene (lanes 3-5) or spontaneously transformed (lane 6); malignant cell line derived from a patient's rhabdosarcoma (lane 7), osteosarcoma (lanes 8 and 9), cervical carcinoma (lane 10), bladder carcinoma (lane 11), neurofibrosarcoma (lane 12); the normal cell line from which MSU-1.1 cells are derived (lane 13); parental MSU-1.1 cells (lane 14); malignant cell lines derived from a patient's fibrosarcoma (lanes 15-19), or neurofibrosarcoma (lane 20); and malignant cell lines derived from athymic mice tumors formed after injection of cells transformed by cobalt 60, viz., MSU-1.1 cells (lane 21), MSU-1.2 cells (lanes 22 and 23). The KD1 cell line was originally obtained from the late Dr. Takeo Kakunaga. The other cell lines are described in Table 1. (B) RNA loading evaluated by a GAPDH cDNA probe.

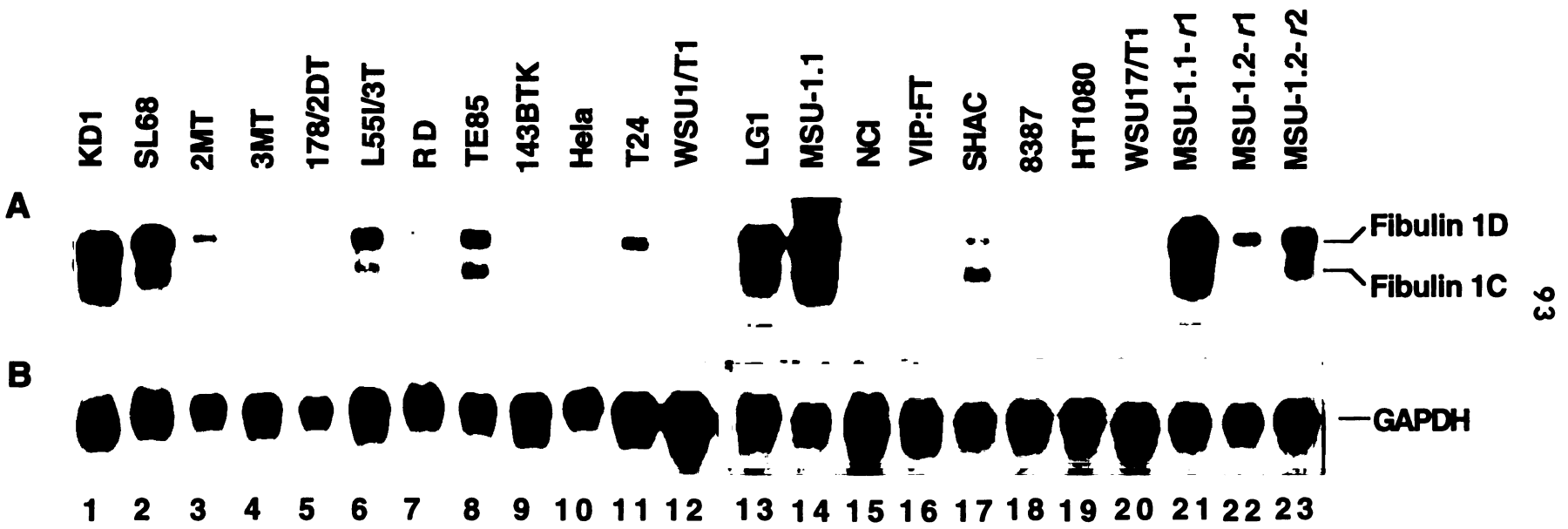
KD1
SL68
2MT
3MT
178/2DT
L55I/3T
RD
TE85
143BTK
Hela
T24
WSU1/T1
LG1
MSU-1.1
NCI
VIP:FT
SHAC
8387
HT1080
WSU17/T1
MSU-1.1-r1
MSU-1.2-r1
MSU-1.2-r2
A
B
Fibulin 1D
Fibulin 1C
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

Fig. 2

reduction in the size of colonies was seen for 6A/SB1 cell transfectants. These results indicate that elevated expression of fibulin-1D can partially suppress anchorage independence in the fibrosarcoma-derived cell lines tested.

In vitro invasiveness of fibulin-1D transfectants

To analyze the invasive potential of the fibulin-1D transfectants, we used a modified Boyden Chamber Matrigel assay. As shown in Figure 6, the ability of the fibulin-1D expressing transfectants to reach the bottom of the filters through the matrigel was reduced by 50-60% compared to that of parental cells and the vector-transfected control cell strains of both SHAC and 6A/SB1 cells.

In vivo tumorigenicity of the fibulin-1D-expressing clones

To determine whether restoration of the expression of the fibulin-1D gene would also suppress in vivo tumor growth, we inoculated the fibulin-1D-expressing transfectant cell strains, their nontransfected parental cell lines, and vector-transfected controls into athymic mice and monitored the animals for growth of tumors. As shown in Figure 7, the latency of tumor formation for the fibulin-1D-expressing clones was longer than that of the parental cells and the vector-transfected control cell strains. For SHAC cells and their vector-transfected control cell strains, the tumor volume reached 500 mm^3 after an average of 25 days postinjection; whereas for fibulin-1D-transfected cells, the average time was 55 days. For 6A/SB1 cells and their vector-transfected control strains, with the exception of one, the tumor volume reached 500 mm^3 after an average of 20 days postinjection; whereas the average time for four fibulin-1D-expressing clones was 48 days, and the other two fibulin-1D-expressing clones failed to form tumors in three months.

In vitro proliferation of fibulin-1D transfectants

Fig. 3. Western blot analysis of the expression and secretion of fibulin-1 by normal cells (LG1 and MSU-1.1) and 10 tumor-derived cell lines under non-reducing conditions. Cellular protein (20 μg) (A) and 50 μl of the medium conditioned by the same cell lines (B) were analyzed by electrophoresis in 10% polyacrylamide gel and probed with a monoclonal antihuman fibulin-1 antibody 3A11. The positive control (lane 1) was fibulin-1 protein isolated and purified from human placenta; lanes 4-8 are from malignant cell lines derived from human fibrosarcomas from patients; lane 9 from a rhabdomyosarcoma; lane 10 from an osteosarcoma; lanes 11-13 are from malignant cell lines derived from tumors in athymic mice from carcinogen-transformed MSU-1.1 cell strains.

A
B
Fibulin 1
LG1
MSU-1.1
NCI
VIP:FT
SHAC
8387
HT1080
RD
TE85
6A/SB1
3MT
1.2- M/SF1
—80 KD
—80 KD

Fig.3

If the transfected cells expressing fibulin-1D grew more slowly than the parental cells, this could explain the biological data described above. To determine the doubling time of each of the fibrosarcoma-derived cell lines, the parental cells, two vector control transfectants, and three fibulin-1D-expressing clones were allowed to grow exponentially and the number of cells was determined at several time points (Table 2). For SHAC cell line and its clonal derivatives, there was no difference in the growth rate of the vector-transfected control cell strains and that of the fibulin-1D-expressing cell strains. For cell line 6A/SB1 and its clonal derivatives, introduction of fibulin-1D had no consistent effect on the growth rate of these cells. The three fibulin-1D expressing clones grew somewhat more slowly (23.8-25.8 h per doubling) than the parental cells (20.2 h). One of the vector control clones, CON3, also grew at this slower rate (25.7 h), but did not exhibit a longer than normal tumor latency period. Therefore, the lengthened latency period seen in Figure 7 cannot be accounted for by a slower growth rate of the fibulin-1D-transfectant clones.

Fig. 4. Western blot analysis of the expression and secretion of fibulin-1D protein by Fibulin-1D transfectants. Whole cell lysates and conditioned medium from (A) LG1cells and 6A/SB1 cells, three vector-transfected controls (CON1, CON2 and CON3) and six fibulin-1D-expressing transfectants of 6A/SB1 cells and (B) SHAC cells, two vector-transfected controls (8a1 and 8b2) and five fibulin-1D-expressing transfectants of SHAC cells were assayed. Cellular protein (20 μg) (upper panel) and 50 μl of the media conditioned by these cell lines (lower panel) were subjected to immunoblotting analysis as described in Figure 3. An arrow indicates the dimer of fibulin-1D, and an arrowhead indicates the monomer.

A
203 KD
116 KD
86 KD
51 KD
LG1
6A/SB1
CON1
CON2
CON3
2 b
2 c
5 c
8 b
10 b
11 a
ω
SHAC
8a1
8b2
5a3
5c4
5d2
5e1
5f3

Fig.4

Fig. 5. Colony formation by fibulin-1D transfected cell strains in 0.33% agarose. Cell strains used were: 6A/SB1 cells (A), its vector-transfected control clone CON3 (B) and its fibulin-1D-expressing clone 2b (C) and clone 11a (D), SHAC cells (E), its vector-transfected control clone 8a1(F) and its fibulin-1D-expressing clone 5a3 (G) and clone 5c4 (H). For each cell strain 2 x 10^4 cells were plated in agarose at 5,000 cells per 60 mm-diameter dish. After 2 weeks (A-D) or 3 weeks (E-G) of incubation, a photomicrograph of a representative field was taken from each dish. Representative examples are shown.

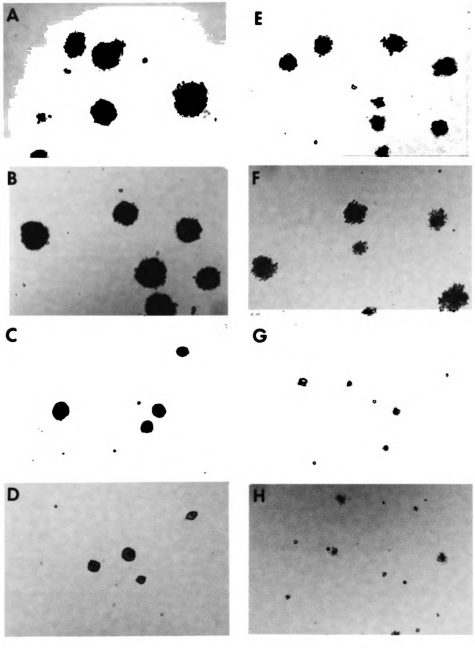

Fig. 5

Table 2 Growth properties of parental cells, cells expressing a transfected-fibulin-1D gene, and vector-transfected control cells

Cell Strains or lines	Number of colonies of a given size per 5,000 cells assayed in agarose		Population doubling time (h) mean ± SD	Cell strains or lines	No. of colonies with a diameter ≥ 100μm per 5,000 cells assayed	Population doubling time (h) mean ± SD
	Diameter ≥ 150 μm	Diameter ≥ 200 μm				
6A/SB1	1250	800	16.7 ± 2.1	SHAC	1000	18.4 ± 0.5
CON1	1440	730	16.6 ± 1.6	8a1	1280	23.2 ± 2.1
CON2	1300	700	ND[a]	8b2	1920	22.9 ± 2.2
CON3	930	740	23.5 ± 4.7	5a3	30	22.1 ± 1.1
2b	130	0	27.3 ± 4.6	5c4	0	19.5 ± 1.8
2c	100	0	ND	5d2	30	22.6 ± 2.0
5c	60	0	ND	5e1	60	ND
8b	990	290	24.1 ± 2.4	5f3	0	ND
10b	210	0	ND			
11a	30	0	23.2 ± 2.2			

[a] Not determined.

DISCUSSION

In the present study, using mRNA differentially display, we found that the low expression of an extracellular matrix and glycoplasma protein, fibulin-1D, is associated with malignant transformation. High levels of fibulin-1 expression have been reported in a wide spectrum of normal human tissues and organs (Zhang et al., 1994). In the experiments described here, we assayed 15 tumor cell lines derived from human cancer patients and found that 12 cell lines showed very low levels of fibulin-1 expression. Since the normal cells from which these tumor cells were derived are not available, we cannot be certain that the low expression is the result of downregulation. However, we observed that seven out of 16 MSU-1.1-derived tumor cell lines exhibited markedly reduced levels of fibulin-1 expression compared with the parental MSU-1.1 cells, indicating that down regulation of fibulin-1 expression occurs relatively frequently in the transformation of human fibroblasts in culture. Our data also showed that fibulin-1D downregulation is not specifically correlated with the transformation induced by particular oncogenes or by specific carcinogens. Southern blotting analysis revealed that the downregulation of fibulin-1D in the MSU-1.1 cell strain derivatives cannot be attributed to gross genetic deletions or rearrangements (data not shown).

Fibulin-1, together with fibulin-2, belongs to a family of extracellular matrix proteins (Pan et al., 1993a; Tran et al., 1997). Alternative splicing of fibulin-1 precursor RNA results in four transcripts that encode polypeptides differing from each other only at the carboxyl terminal regions. The four isoforms are designated A-D. The dominant forms expressed in most human tissues and cell lines in culture are fibulin-1C and fibulin-1D (Zhang et al., 1994; Tran et al., 1997). Fibulin-1 proteins are multimodular proteins containing three cysteine-rich anaphylatoxin-related segments, nine epidermal growth factor-like repeats with a consensus motif for calcium-binding, and a variable carboxyl-

Fig. 6. In vitro invasiveness of fibulin-1D transfected cell strains in matrigel assays. The invasive index for each cell strain was determined from the number of cells that passed through the matrigel inserts as a fraction of the number of cells that passed through the control uncoated filters. The invasion index of SHAC and 6A/SB1 cells was normalized to 100.

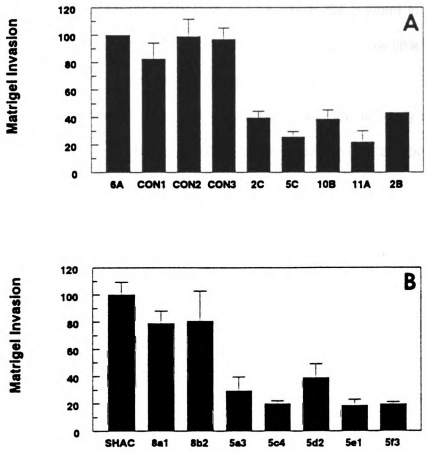
Matrigel Invasion
120
100
80
60
40
20
0
A
6A
CON1
CON2
CON3
2C
5C
10B
11A
2B
Matrigel Invasion
120
100
80
60
40
20
0
B
SHAC
8a1
8b2
5a3
5c4
5d2
5e1
5f3

Fig. 6

terminal domain (Argraves et al., 1990; Pan et al., 1993b). Biochemical studies have shown that fibulin-1 interacts with itself and binds other extracellular matrix proteins, including fibronectin (Balbona et al., 1992), laminin, and nidogen (Pan et al., 1993b). Immunohistochemical studies have shown that fibulin-1 proteins are widely expressed intercellular components of connective tissues which are present in matrix fibers and basement membranes (Roark et al., 1995). However, until now little was known about fibulin-1 playing a role in the process of transformation.

Since we found that the expression of fibulin-1 was low in many tumor-derived cell lines, we sought to determine whether this low expression of fibulin-1 is merely a consequence of malignant transformation, or whether it plays a role in the malignant transformation process. Our results with a transfected fibulin-1D gene demonstrate that in each case (11 out of 11), elevated expression of fibulin-1 can suppress the ability of fibrosarcoma-derived cells to proliferate in soft agar and to delay the tumorigenicity in vivo. The mechanisms underlying the observed effects have not yet been elucidated. However, a number of studies regarding the role of other ECM molecules in transformation indicate that there is a good correlation between the loss of specific ECM molecules and the acquisition of transformed phenotypes in vitro and tumorigenicity in vivo. For instance, hybrid cells formed by fusion of mouse melanoma cells and normal mouse fibroblasts are not malignant. However, inhibition of fibronectin synthesis in such hybrids by an antisense strategy causes the cells to become tumorigenic (Steel and Harris, 1989). Conversely, addition of purified fibronectin to Herpes simplex virus-transformed hamster fibroblasts can restore normal morphology and adhesive properties characteristic of normal cells (Ali et al., 1977). Overexpression of fibronectin in human fibrosarcoma-derived HT1080 cells with increased deposition of fibronectin on the cell surface leads to reduced anchorage-independent growth and tumorigenicity

Fig. 7. Tumorigenecity of fibulin-1D transfected cell strains in athymic mice. The cell strains used were: SHAC cells (A), 6A/SB1 cells (B), their control vector or fibulin-1D transfectants. For each cell line, two mice (total four sites) were injected with 1 x 10^6 cells at each site. Each symbol represents the volume averaged from four tumors from two mice: parental cell lines (•), control vector transfectants (other closed symbols), and fibulin-1D transfectants (open symbols).

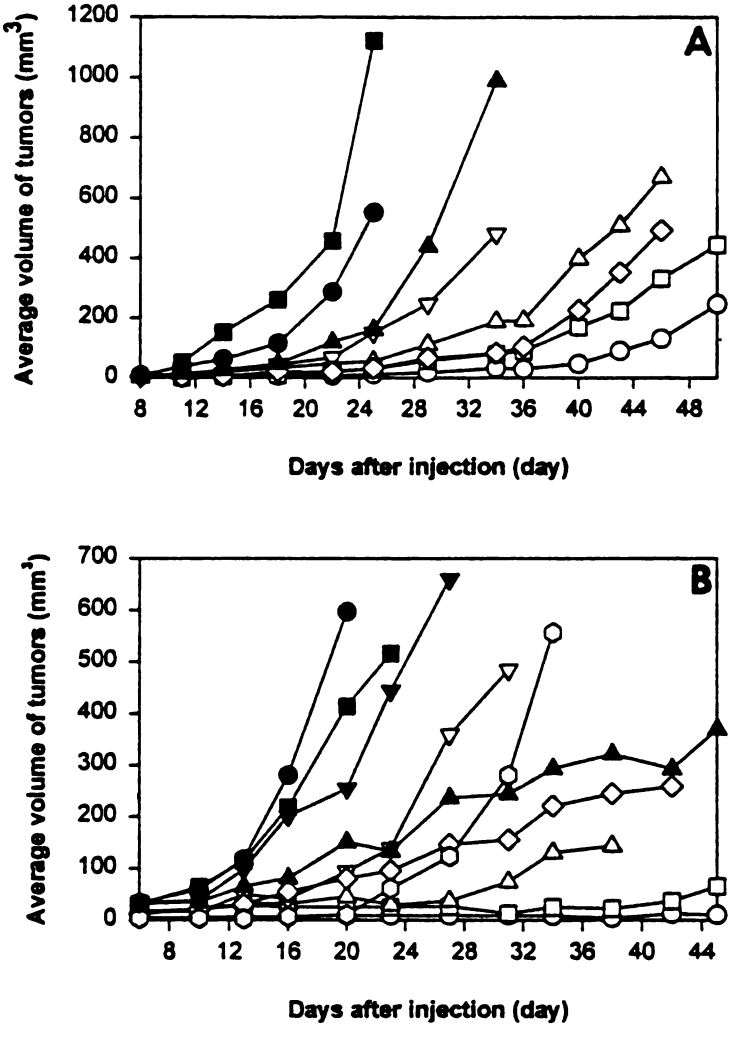
A
Average volume of tumors (mm3)
1200
1000
800
600
400
200
0
8
12
16
20
24
28
32
36
40
44
48
Days after injection (day)
B
Average volume of tumors (mm3)
700
600
500
400
300
200
100
0
8
12
16
20
24
28
32
36
40
44
Days after injection (day)

Fig. 7

(Akamatsu et al., 1996). A recent study shows that expression of SPARC, an ECM protein, in ovarian cancer cells results in a significant decrease in cancer cell growth and tumorigenic potential (Mok et al., 1996). Collectively, these data demonstrate that certain ECM molecules play an important role in the regulation of malignant transformation. Perturbation in expression of these molecules could lead to global effects by interfering with the formation and stabilization of extracellular matrix structures, or as suggested by Juliano and Haskill (1993), with normal cell growth signaling pathways that act through extracellular matrix.

An important conclusion that can be drawn from our results is that loss of fibulin-1D plays a role in invasion and perhaps metastasis. One possible model is that fibulin-1D negatively regulates the ability of cells to interact with migration-promoting ECM components, thereby inhibiting cellular migration. The ability of fibulin-1 to interact with several adhesive and migration-promoting proteins such as fibronectin and laminin has been established (Balbona et al., 1992; Pan et al., 1993b). It is possible that fibulin-1 binding to these proteins interferes with the ability of cells to derive migratory stimuli. We know that the fibulin-1 binding site in fibronectin is located within type III repeats 13 and 14 (Balbona et al., 1992). This region lies adjacent to the Arg-Gly-Asp (RGD)-containing type III_{10} adhesive domain and the CSIII adhesive domain (Argraves and Gehlsen, 1991). These domains mediate cellular interaction with fibronectin via a wide array of integrins, including $\alpha5\beta1$ binding to the RGD site and $\alpha4\beta1$ binding to the CSIII site. It is possible that the binding of fibulin-1 to fibronectin interferes with integrin binding to either or both of these sites. The fibulin-1 binding site with Engelbreth-Holm-Swarm tumor laminin has been mapped to a site contained within the E3 fragment (Pan et al., 1993b; Brown et al., 1994), a fragment derived from the carboxy terminus of the á chain. This domain has been shown to mediate integrin $\alpha3\beta1$binding (Gehlsen et al.,

1989; Gehlsen et al., 1992) and lies in the vicinity of binding sites for the integrins α6β1 (Aumailley et al., 1990; Hall et al., 1990; Sonnenberg et al., 1991) and α7β1 (Kramer et al., 1991; von der Mark et al., 1991). In addition, the laminin receptor dystroglycan binds to the E3 fragment (Gee et al., 1993). Studies are under way to determine whether fibulin-1 modulates any of these receptor-adhesive protein interactions.

Interestingly, in contrast to our findings, Clinton et al. (1996) recently reported that estrogens increase the expression of fibulin-1 in human ovarian cancer cells. They suggest that the augmented fibulin-1 expression facilitates ovarian tumor cell invasion. However, they have not yet carried out studies to test this hypothesis. It may also be that estrogen-augmented fibulin-1 expression acts to inhibit tumor cell migration. Clearly, additional experiments are required to determine the role that fibulin-1 plays in the movement and growth of various types of tumor cells.

In summary, by using differential mRNA display we have identified fibulin-1 as a gene which is downregulated in a human fibroblastic cell strain transformed in culture. We have found that the fibulin-1D protein is expressed at a low or completely undetectable level in a number of human fibroblastic cells transformed in culture as well as in a number of human tumor-derived cell lines. We have demonstrated that increased expression of fibulin-1D from a transfected gene in human fibrosarcoma-derived cell lines reduced anchorage-independent growth and delayed tumor growth in athymic mice. Furthermore, the invasive ability of these cells was greatly suppressed. These findings indicate that the loss of fibulin-1D expression contributes to the transformation and progression progress of human fibrosarcoma. This study points to the importance of recent studies that have shown that ECM molecules can directly regulate critical cellular process such as growth, differentiation and apoptosis (Jones et al., 1993; Lin and Bissell, 1993; Meredith et al., 1993; Frisch and Francis, 1994). Further research in

various types of cells will be required to address the precise mechanism by which ECM contributes to the transformation process.

MATERIALS AND METHODS

Cells and cell culture

The infinite life span human fibroblast cell strain MSU-1.1 and its derivative cell lines were routinely cultured in Eagle's minimum essential medium, modified by addition of L-aspartic acid (0.2 mM), L-serine (0.2 mM) and pyruvate (1mM) and supplemented with 10% supplemented calf serum (SCS) (Hyclone Laboratory, Logan, UT), penicillin (100 units/ml), streptomycin (100 μg/ml) and hydrocortisone (1 μg/ml) (complete medium) at 37°C in a humidified incubator containing 5% CO_2 in air.

Growth rate.

Growth curves were obtained by plating 2 x 10^4 cells per 60 mm diameter dish and harvesting triplicate dishes on day 1, 3, 4, 5 and 7. The total number of cells per dish was determined using an Elzone Counter. The exponential growth phase of the growth curve was used to calculate the population doubling time.

Differential mRNA display

Non-tumorigenic human fibroblast cell strain MSU-1.1 and its malignant derivative cell line 6A/SB1 were used as sources of RNA. Total RNA from cells in exponential growth was extracted using RNAzolB (Tel-Test, Friendswook, TX). Differential mRNA display was performed as described (Liang and Pardee, 1992; Liang et al., 1993) with slight modifications.

Briefly, total RNA (0.2 μg) from each cell line was reverse transcribed with each of the four degenerate oligo (dT) primers $T_{12}MA$, $T_{12}MT$, $T_{12}MG$ and $T_{12}MC$ (where M may be dG, dC, or dA), followed by PCR amplification of the cDNA in the presence of [α-^{35}S] dATP (Dupont, Wilmington, DE) using the corresponding $T_{12}MN$ as the 3' primer and one of the arbitrary 10-mers (OPA1-20, Operon Technologies, Alameda, CA) as the 5' primer. The PCR thermal cycle parameters were as follows: 94°C for 30 s, 40°C for 2

min, and 72°C for 30 s for 40 cycles, followed by extension at 72°C for 7 min. PCR products were separated in a 6% denaturing polyacrylamide gel. Gels were dried without fixation and exposed to Kodak XAR film. To confirm the results, reactions showing differentially expressed mRNAs in MSU-1.1 and 6A/SB1 cells were repeated in duplicate using two independent RNA preparations. The cDNA fragments representing uniquely expressed mRNAs were excised from the dried gels and reamplified by PCR using the same set of primers originally used. The PCR products were run on a 1.5% agarose gel, and the bands of the appropriate size were cut from the gel and purified using QIAquick gel extraction kit (Qiagen, Chatsworth, CA).

Subcloning and DNA sequencing

The purified DNA was used as probes for Northern blot analysis or subcloned into the pCRII vector by the TA cloning method (Invitrogen, San Diego, CA) according to manufacturer's instructions. Subsequent identification of insert-containing clones was carried out by the dot-blot DNA hybridization procedure described by Callard et al.(1994). The subcloned cDNA inserts were again used as probes for Northern analysis to confirm the differential expression. DNA sequencing was performed directly from the TA cloning vector with the SP6 and the T7 primer using the Fidelity DNA sequencing system (Oncor, Gaithersburg, MD). DNA database searches were performed using the GCG FASTA program (Genetics Computer Group, Madison, WI) or the BLAST program from National Center for Biotechnology Information.

Northern blot analysis

Total RNA (15 μg) was electrophoresed on a denaturing formaldehyde agarose gel (1.2%), transferred to Hybond-N membrane (Amersham, Arlington Heights, IL) by the downward capillary transfer technique (Chomczynski, 1992) using a 20 fold concentration of standard saline citrate buffer, and fixed by UV crosslinking (UV

Stratalinker 2400, Stratagene, La Jolla, CA). DNA probes were radiolabeled by the random primed labeling method (Feinberg and Vogelstein, 1983). Northern hybridization was performed at 42°C overnight in 50% formamide containing 5 x SSPE, 5 x Denhardt's solution, 0.1% sodium dodecyl sulphate (SDS) and 0.1 mg/ml salmon sperm DNA. The blots were then washed twice in 1 x SSPE, 0.1% SDS at 42°C, and a third wash was carried out in 0.25 x SSPE with 0.1 % SDS at 55°C. To strip the membrane for reprobing, the blots were treated with boiling 0.1% SDS solution and allowed to cool to room temperature. Variation in RNA loading per lane was evaluated by probing with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA as the control.

Preparation of cell lysates and conditioned medium

Cells were grown in 100 mm-diameter dishes in Eagles's medium containing 10% SCS until subconfluent. To harvest the cells, the cell monolayer was quickly washed four times with cold phosphate buffered saline (PBS), and the cells were lysed for 20 min on ice in 0.5 ml of RIPA buffer composed of 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA and 0.15 units/ml aprotinin. The cell lysates were collected with a rubber scraper, centrifuged at 14,000 x g for 15 min at 4°C, and the protein concentration of the supernatant was determined using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL) with bovine serum albumin as the protein standard. The cell lysates were used immediately or stored at –80°C until use. To collect conditioned medium, nearly confluent cells cultured in 60 mm-diameter dishes (i.e., ~ 2 x 10^6 cells) were washed four times with Eagle's medium, and incubated for another 48 h in 4 ml of complete medium without serum. The conditioned medium was collected, and PMSF (final concentration, 1 mM) and aprotinin (0.15 units/ml) were

added, and the medium was centrifuged at 14,000 x g for 5 min to remove cell debris. A second duplicate dish for each sample was used to prepare cell lysate as described above, and the protein concentration of the cell extract was used to normalize the volume of conditioned medium used for Western blot analysis.

Western blot analysis of fibulin-1

Aliquots of cell lysates containing 20 μg of protein or 50 μl of the conditioned medium were mixed with the sample buffer (0.05M Tris-HCl, pH6.9, 9% glycerol, 2.3% SDS and 0.1% bromophenol blue) without a reducing agent, and the proteins were separated on a 10% SDS/polyacrylamide gel. The proteins were then electroblotted onto an Immobilion-P membrane (Millipore, Bedford, MA). The blots were blocked for 2 h at room temperature in Tris-buffered saline (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dry milk (blocking solution), and then incubated for 2 h at room temperature with the mouse monoclonal anti-fibulin-1 IgG 3A11 diluted 1:20,000 in the same solution. This antibody was generated as part of an earlier study (Argraves et al., 1990). The blots were washed several times and then incubated with horseradish peroxidase-conjugated goat-anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN) that had been diluted 1:3000 with blocking solution. Enhanced chemiluminescence (Amersham, Arlington Heights, IL) was used according to the manufacturer's recommendations to detect the signal.

Plasmid construction and transfection

A 2.3-kb EcoRI fragment of pBluescript-Fibulin-1D, containing the entire coding sequence of human Fibulin-1D, was inserted into the unique EcoRI cloning site of the mammalian expression vector pBABE-Puro (Morgenstern and Land, 1990) under the control of Moloney murine leukemia virus long terminal repeat, generating the plasmid pPuro-Fibulin1D. The sense orientation of the cloned fragment was confirmed by

restriction mapping. To produce stably transfected cell lines, cells in exponential growth were plated in 100-mm dishes (2 x 10^5 cells/dish). After 18 to 24 h, 2 μg of plasmid DNA was transfected into the cells using lipofectamine (GIBCO, Gaithersburg, MD) according to the manufacturer's instructions. Transfected cells were selectively grown in culture medium containing 0.5 μg/ml puromycin (Sigma, St. Louis, MO) for 2-3 weeks. Drug-resistant clones were randomly selected and subcloned individually for further study.

Anchorage independence assay

For each cell line or strain, 2 x 10^4 cells were plated at 5,000 cells per 60 mm-diameter dish in a top layer of 0.33% agarose in McM medium containing 2% fetal calf serum, essentially as described by Hurlin et al. (1989). The agarose plates were maintained at 37°C in a humidified incubator with 3% CO_2 in air. Each week the medium over the top layer of agarose was removed and fresh McM medium with 2% fetal calf serum was added. At the end of 14 days, 6A/SB1 cells and their transfectants or 21 days for SHAC cells and their transfectants, the cells were fixed with 2.5% glutaraldehyde. Photomicrographs covering 0.7 cm^2 were made of representative fields from each dish. All colonies greater than 100 ìm in diameter were scored and the size of each colony was recorded. The frequency of colonies of a given size in this area from each dish was averaged, and the data were expressed per 5,000 cells assayed.

In vitro invasion assay

The invasiveness of SHAC cells, 6A/SB1 cells and their transfectants was assayed by a modified Boyden Chamber Matrigel method (Albini et al., 1987) according to the manufacturer's instructions (Collaborative Biomedical Product-Becton Dickson, Bedford, MA). The cells to be studied were washed with PBS three times and harvested by short exposure to 5 mM EDTA. Cells were washed once with Eagle's medium, and 7 x 10^5

cells in Eagle's medium containing 0.1 % BSA were seeded onto the control uncoated filters (8 μm pore size) or matrigel-coated filters in Biocoat Matrigel invasion chambers. Eagle's medium (2.5 ml) containing 0.1% BSA and 10 ng/ml platelet-derived growth factor (Sigma) was added to the lower compartment. After incubation at 37°C for 48 h (for 6A/SB1 cells and its transfectants) or 72 h (for SHAC cells and its transfectants), the filters were fixed in ethanol and stained with 1% crystal violet. Cells that invaded the lower surface of the filters were counted under a light microscope. Each assay was done in triplicate.

Tumorigenicity assay

BALB/c athymic mice 6 weeks of age were injected with 1 X 10^6 cells in 0.2 ml of serum-free Eagle's medium subcutaneously in the left front and right hind flank regions. Tumor dimensions were measured twice weekly using a vernier caliper, and the size of tumors was calculated using the formula for the volume of a hemiellipsoid, the geometric figure most nearly approximating the shape of the tumor: Volume=0.5236 X length X width X height (Rockwell et al., 1972).

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CHAPTER III

Cloning and Characterization of a Novel Gene Encoding a Putative Transmembrane Protein with Altered Expression in Human Tumors

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ABSTRACT

Identification and characterization of genes expressed in normal cells and decreased in their malignant counterparts is an important method for detecting candidate tumor suppressors. Using differential display of mRNAs from nontumorigenic infinite life span human fibroblast cell strain MSU-1.1 and an isogenic fibrosarcoma-derived cell line, 6A/SB1, which was derived from chemical carcinogen transformed MSU-1.1 cells, we identified a novel gene, ST7, showing a 6-fold lower expression in 6A/SB1 cells compared with parental MSU-1.1 cells. Molecular cloning of a near full-length cDNA revealed that the novel gene encodes a putative transmembrane protein composed of 859 amino acids: the 492 N-terminal amino acids including a 5-fold cysteine-rich repeat of 40 amino acids homologous to the ligand binding repeat of the known low density lipoprotein receptor, a 24 hydrophobic amino acid stretch spanning the plasma membrane, and a C-terminal domain of 343 residues. The ST7 gene is widely expressed in normal human tissues and is particularly abundant in human heart and skeletal muscle. Northern analysis showed that 10 out of 15 tumor cell lines derived from patients and 6 out of 16 cell lines established from tumors formed in athymic mice by MSU-1.1 cells transformed in culture have low or undetectable levels of ST7 mRNA. Furthermore, Western blotting analysis using a specific anti-peptide antibody demonstrated that the levels of ST7 protein are high in normal fibroblasts and low in 12 sarcoma-derived cell lines tested. Altered expression of ST7 appears to occur at both the transcriptional and posttranscriptional level. These studies make it possible to characterize a novel putative receptor protein, whose expression is downregulated in many malignantly transformed fibroblastic cells, and which may play an important role in the transformation of these cells.

INTRODUCTION

Cancer results from the accumulation of a series of genetic and biochemical changes in normal cells (Peto, 1977; Farber, 1984; Klein and Klein 1985; Fearon and Vogelstein, 1990). Despite the vast increase in our knowledge of oncogenes and tumor suppressor genes associated with human neoplasia over the past 15 years, the molecular events leading to the formation of most types of human tumors are still not well understood. Therefore, identification of genes that are involved in the transition of non-tumorigenic cells to malignant cells can be expected to help us understand the molecular mechanisms underlying tumor formation.

The recent introduction of several in vitro transformation systems utilizing human cells in culture (Stoner et al., 1991; Reznikoff et al., 1993; McCormick and Maher, 1994; Rhim et al., 1994; Park et al., 1995) has facilitated the investigation of the cellular and molecular mechanisms involved in the multistep carcinogenic process. In our laboratory, transfection of the v-myc oncogene into a human neonatal foreskin-derived fibroblast cell line LG1 led to the establishment of a near-diploid, karyotypically stable, infinite life span human fibroblast cell strain MSU-1.1 (Morgan et al., 1991). MSU-1.1 cells are phenotypically normal and do not form tumors in athymic mice. Treatment of MSU-1.1 cells with chemical carcinogens such as benzo(a)pyrene diol epoxide (BPDE) (Yang et al., 1992) or gamma irradiation (Reinhold et al., 1996), followed by selection of focus-forming cells, results in cells capable of forming tumors in athymic mice. Since the focus-derived, tumorigenic cells have various properties not possessed by the parental cells, it is presumed that additional genetic alterations induced by carcinogens are required in the transformation process. However, the cellular genes responsible for the neoplastic transformation induced by these carcinogens remain poorly understood.

The present study was carried out seeking to isolate the oncogene(s), or tumor suppressor gene(s) that are involved in this neoplastic conversion.

The recently developed differential mRNA display method (Liang and Pardee, 1992; Liang et al., 1993) allows one to identify genes that are differentially expressed between closely related eukaryotic cells. In our study, we applied this method to compare the mRNA profile between the nontumorigenic parental MSU-1.1 cells and one of its malignant derivative cell lines, 6A/SB1, which was established from a fibrosarcoma formed in an athymic mouse by BPDE-transformed MSU-1.1 cells. We have identified a novel gene, designated ST7, whose mRNA is markedly downregulated in 6A/SB1 cells compared with MSU-1.1 cells. A near full-length cDNA was cloned and the deduced amino acids revealed that this novel gene encoded a putative transmembrane protein of 859 amino acids. ST7 mRNA and protein levels were low in a large fraction of the tumor derived cell lines tested. Further characterization of ST7 should lead to better understanding of the carcinogenesis process in human cells.

RESULTS

Identification of ST7 as a Gene Differentially Expressed Between Preneoplastic and Malignant Human Cells. When we compared MSU-1.1 cell strain and its tumorigenic derivative cell line 6A/SB1 using mRNA differential display, we saw 8,000-10,000 displayed cDNA fragments and found in two independent experiments that nine DNA fragments reproducibly showed differential intensities between MSU-1.1 cells and the tumor-derived cells (Figure 1A and data not shown). cDNA fragments were isolated from the sequencing gel and used as probes in Northern analysis to test for differential mRNA expression between MSU-1.1 and 6A/SB1 cells. Differential expression of mRNA was confirmed for five of the nine cDNAs. Subsequent analysis showed that four of these five corresponded to the same gene, i.e., fibulin-1D (Qing et al., 1997). The fifth, designated ST7, is shown in Figure 1B. This cDNA fragment hybridized with a 3.7 kb transcript showing a 6-fold lower expression in 6A/SB1 cells than in MSU-1.1 cells. The ST7 DNA fragment was subcloned, and the insert DNA from several individual plasmids was also found to hybridize to the same sized RNA and the transcript exhibited differential expression in MSU-1.1 cells compared with the tumor cell line. Subsequent sequencing analysis of this partial cDNA revealed that it contained 485 bp with no significant homology with any known genes in the nucleotide sequence databases.

Expression of ST7 in Multiple Human Tumor Cell Lines. To test whether the expression of ST7 mRNA is also downregulated in other tumor-derived MSU-1.1 cell lines malignantly transformed by various methods, RNA from 15 additional such cell lines was assayed by Northern blotting analysis. We also examined 15 tumor-derived cell lines from patients. The origin of these cell lines has been described previously (Qing et al., 1997). Representative data are shown in Figure 2. We found

downregulation of ST7 in 5 out of 15 MSU-1.1 derivatives (Lanes 4, 5, 8, 21 and 22). We also found that in 10 out of 15 tumor-derived cell lines from patients, the expression of ST7 was very low or undetectable, i.e., in cells from three out of five fibrosarcomas (Lanes 10, 12-13), in cells from two out of two osteosarcomas (Lanes 6 and 16), in cells from the three neurofibrosarcomas (Lanes 18-20), and in cells from a cervical carcinoma (Lane 7) and a bladder carcinoma (Lane 3). Collectively, of the 31 tumor-derived cell lines assayed, 16 exhibited either low or no expression of ST7.

Molecular Cloning of the Full-length Human ST7 cDNA. To obtain the full-length cDNA of ST7, we screened a human fibroblast cDNA library (library 9 of Legerski) with an ST7 gene-specific primer and a vector-specific primer, using the High Fidelity Expand polymerase chain reaction method (Boehringer Mannheim). Several specific PCR products were obtained. We sequenced the longest one (clone A), which was about 2.6 kb (Figure 3A). To recover the 5' end of the cDNA, we screened a human skeletal muscle cDNA library with another ST7-specific primer and a vector primer using the PCR method described above. This produced a fragment we called clone B. To obtain additional 5' sequence, we carried out the 5'-rapid amplification of cDNA ends (RACE) reaction using a human heart Marathon-ready cDNA (Clontech, Palo Alto, CA) and designated the generated fragment clone C. These three clones overlapped with each other (Figure 3A). Two forms of the cDNA differing from each other at the 5' end were isolated. The longer form has 57 more nucleotides than the shorter one. The assembled nucleotide sequence of the longer form (total 3078 bp) and the deduced amino acid sequence are shown in Figure 3B. An open reading frame extends from nucleotide 43 to 2619 and encodes a protein of 859 amino acids with an estimated molecular weight of 92.8 kDa. The first ATG codon (nucleotides 43-45) lies in the context of the Kozak

Fig.1. (A) Differential display comparing mRNAs from the parental MSU-1.1cell strain (lanes 1 and 2) and its tumorigenic derivative cell line 6A/SB1 (lanes 3 and 4). For each cell line, two independent preparations of total RNA were extracted, reverse transcribed, and amplified by PCR in the presence of [α-^{35}S]dATP. The PCR products were separated on a 6% polyacrylamide gel and autoradiographed. The signal demonstrating altered expression is marked by an arrow. The primers used were T_{12}MT and OPA7 as described by Qing et al. (1997). (B) Northern blot analysis confirming differential gene expression for fragment ST7 using the amplified DNA fragment as a probe. Total RNA (15 μg) obtained from MSU-1.1 cells (lane 1) and 6A/SB1 cells (lane 2) was subjected to Northern analysis as detailed in "Materials and Methods". The blot was stripped and reprobed with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (lower panel) as the loading control.

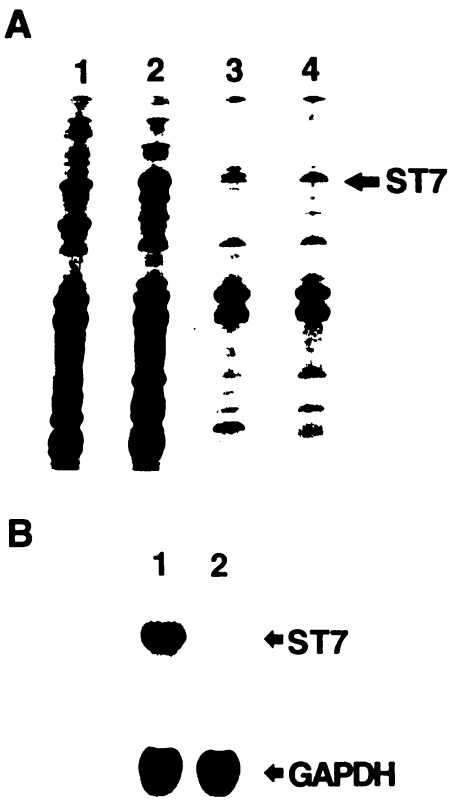

Fig.1

consensus initiation site of eukaryotic mRNA translation (Kozak, 1991). The ST7 cDNA contains a putative 3' untranslated region of 450 bp followed by a poly A tail and three consensus polyadenylation signals (Wahle and Keller, 1992) at nucleotide positions 2846, 2976 and 3034. Comparison of ST7 cDNA sequence to the Genbank and EMBO database using the FASTA and the BLAST program revealed that there is no significant homology with any known genes.

Primary Structure Suggests a Novel Transmembrane Protein. Comparison of the deduced amino acid sequence of ST7 with a nonredundant protein sequence database revealed that overall there was no significant homology with other proteins. A striking feature of this protein is that the amino-terminus is composed of five imperfect repeats of a 40-amino acid cysteine-rich repetitive sequence homologous to that found in the human low density lipoprotein (LDL) receptor (Shdhof et al., 1985) (Figure 4) and complement protein C9 (Stanley et al., 1985). The similarity also includes the highly conserved, negatively charged Ser-Asp-Glu triad, which occurs near the COOH-terminal end of each repeat. Hydropathy analysis (Kyte and Doolittle, 1982) revealed that the coding sequence contained a stretch of 24 hydrophobic amino acids extending from residues 493 to 516 (underlined in Figure 3B), which is flanked at both ends by positively charged residues. This hydrophobic sequence resembles the membrane-spanning region of other transmembrane proteins (Sabatini et al., 1982). The predicted protein also contains other putative functional domains, including nine putative N-linked glycosylation sites, a number of potential phosphorylation sites for protein kinase C and casein kinase II, and several N-myristoylation sites. The importance of these putative domains of ST7 protein under physiological conditions remains to be determined.

Fig. 2. Northern blot analysis of ST7 expression in multiple human tumor cell lines. (A) Total RNA (15 μg), obtained from a series of cell lines, was hybridized with ST7 cDNA using a cloned ST7 DNA fragment as a probe. These were: fibroblast cell line from a normal donor (lane 1); parental MSU-1.1 cells (lanes 2 and 9); malignant cell lines derived from a patient's cervical carcinoma (lane 3); malignant cell lines derived from tumors formed in athymic mice after injection of MSU-1.1 cells transformed by a transfected oncogene (lane 4) or by carcinogen treatment (lane 5); malignant cell lines derived from a patient's osteosarcoma (lanes 6 and 16), bladder carcinoma (lane 7); malignant cell lines derived from tumors formed in athymic mice after injection of MSU-1.1 cells spontaneously transformed (lane 8); malignant cell lines derived from a patient's fibrosarcoma (lanes 10-14), rhabdomyosarcoma (lane 15), Wilms' tumor (lane 17), and neurofibrosarcoma (lanes 18-20); and malignant cell lines derived from tumors formed in athymic mice after injection of MSU-1.2 cells transformed by cobalt 60 (lanes 21 and 22). (B) RNA loading, evaluated by rehybridizing the same blots with a GAPDH cDNA probe.

A
B
SL89
MSU-1.1
Hela
178-2DT
11C/SB1
143BTK
T 24
L46l/7T
MSU-1.1
NCI
VIP:FT
SHAC
8387
HT1080
R D
TE85
GM3808
NF1/FT1
WSU1/T1
WSU17/T1
MSU-1.2-r1
MSU-1.2-r2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Fig.2

Fig. 3. Characterization of ST7 cDNA clones. (A) A diagram showing the four overlapping ST7 cDNA clones. They were isolated from MSU-1.1 cells, a human fibroblast library, a skeletal muscle cDNA library, and a human heart Marathon-ready cDNA, as described in Materials and methods. (B) A near full-length cDNA sequence of ST7 gene and the deduced amino acid sequence. The amino acid sequence is numbered beginning with the first methionine of the longest open reading frame. The one-letter code is used for amino acids. The 19 amino acids existing only in the longer isoform is indicated by double underlining. The putative transmembrane domain is underlined. The peptide used for antibody generation is in bold font. The putative polyadenylation signals are indicated with sets of asterisks.

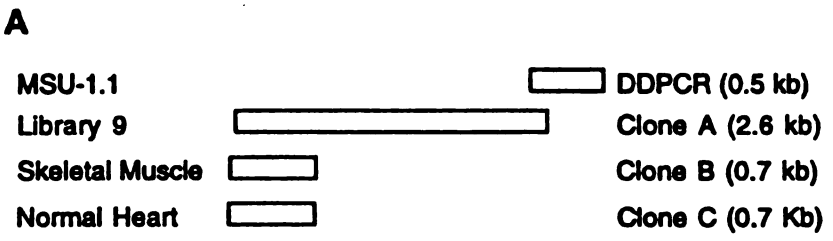
A
MSU-1.1
DDPCR (0.5 kb)
Library 9
Clone A (2.6 kb)
Skeletal Muscle
Clone B (0.7 kb)
Normal Heart
Clone C (0.7 Kb)

Fig. 3

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CTCCTCCTCCGTCTCCTCCTCTCTCTCTCATCTGCTGTGGTTATGGCCTGTCGCTGGAGCACAAAAGAGTCTCCGCGGTGGAGGTCTGCGTTGCTCTTGCTTTTCCTCGCTGGGGTGTAC  120
                                                  M  A  C  R  W  S  T  K  E  S  P  R  W  R  S  A  L  L  L  L  F  L  A  G  V  Y   26

GGAAATGGTGCTCTTGCAGAACATTCTGAAAATGTGCATATTTCAGGAGTGTCAACTGCTTGTGGAGAGACTCCAGAGCAAATACGAGCACCAAGTGGCATAATCACAAGCCCAGGCTGG  240
G  N  G  A  L  A  E  H  S  E  N  V  H  I  S  G  V  S  T  A  C  G  E  T  P  E  Q  I  R  A  P  S  G  I  I  T  S  P  G  W   66

CCTTCTGAATATCCTGCAAAAATCAACTGTAGCTGGTTCATAAGGGCAAACCCAGGCGAAATCATTACTATAAGTTTTCAGGATTTTGATATTCAAGGATCCAGAAGGTGCAATTTGGAC  360
P  S  E  Y  P  A  K  I  N  C  S  W  F  I  R  A  N  P  G  E  I  I  T  I  S  F  Q  D  F  D  I  Q  G  S  R  R  C  N  L  D  106

TGGTTGACAATAGAAACATACAAGAATATTGAAAGTTACAGAGCTTGTGGTTCCACAATTCCACCTCCGTATATCTCTTCACAAGACCACATCTGGATTAGGTTTCATTCGGATGACAAC  480
W  L  T  I  E  T  Y  K  N  I  E  S  Y  R  A  C  G  S  T  I  P  P  P  Y  I  S  S  Q  D  H  I  W  I  R  F  H  S  D  D  N  146

ATCTCTAGAAAGGGTTTCAGACTGGCATATTTTTCAGGGAAATCTGAGGAACCAAATTGTGCTTGTGATCAGTTTCGTTGTGGTAATGGAAAGTGTATACCAGAAGCCTGGAAATGCAAT  600
I  S  R  K  G  F  R  L  A  Y  F  S  G  K  S  E  E  P  N  C  A  C  D  Q  F  R  C  G  N  G  K  C  I  P  E  A  W  K  C  N  186

AACATGGATGAATGTGGAGATAGTTCCGATGAAGAGATCTGTGCCAAAGAAGCAAATCCTCCAACTGCTGCTGCTTTTCAACCCTGTGCTTACAACCAGTTCCAGTGTTTATCCCGTTTT  720
N  M  D  E  C  G  D  S  S  D  E  E  I  C  A  K  E  A  N  P  P  T  A  A  A  F  Q  P  C  A  Y  N  Q  F  Q  C  L  S  R  F  226

ACCAAAGTTTACACTTGCCTCCCCGAATCTTTAAAATGTGATGGGAACATTGACTGCCTTGACCTAGGAGATGAGATAGACTGTGATGTGCCAACATGTGGGCAATGGCTAAAATATTTT  840
T  K  V  Y  T  C  L  P  E  S  L  K  C  D  G  N  I  D  C  L  D  L  G  D  E  I  D  C  D  V  P  T  C  G  Q  W  L  K  Y  F  266

TATGGTACTTTTAATTCTCCCAATTATCCAGACTTTTATCCTCCTGGAAGCAATTGCACCTGGTTAATAGACACTGGTGATCACCGTAAAGTCATTTTACGCTTCACTGACTTTAAACTT  960
Y  G  T  F  N  S  P  N  Y  P  D  F  Y  P  P  G  S  N  C  T  W  L  I  D  T  G  D  H  R  K  V  I  L  R  F  T  D  F  K  L  306

GATGGTACTGGTTATGGTGATTATGTCAAAATATATGATGGATTAGAGGAGAATCCACACAAGCTTTTGCGTGTGTTGACAGCTTTTGATTCTCATGCACCTCTTACAGTTGTTTCTTCT  1080
D  G  T  G  Y  G  D  Y  V  K  I  Y  D  G  L  E  E  N  P  H  K  L  L  R  V  L  T  A  F  D  S  H  A  P  L  T  V  V  S  S  346

TCTGGACAGATAAGGGTACATTTTTGTGCTGATAAAGTGAATGCTGCAAGGGGATTTAATGCTACTTACCAAGTAGATGGGTTCTGTTTGCCATGGGAAATACCCTGTGGAGGTAACTGG  1200
S  G  Q  I  R  V  H  F  C  A  D  K  V  N  A  A  R  G  F  N  A  T  Y  Q  V  D  G  F  C  L  P  W  E  I  P  C  G  G  N  W  386

GGGTGTTATACTGAGCAGCAGCGTTGTGATGGGTATTGGCATTGCCCAAATGGAAGGGATGAAACCAATTGTACCATGTGCCAGAAGGAAGAATTTCCATGTTCCCGAAATGGTGTCTGT  1320
G  C  Y  T  E  Q  Q  R  C  D  G  Y  W  H  C  P  N  G  R  D  E  T  N  C  T  M  C  Q  K  E  E  F  P  C  S  R  N  G  V  C  426

TATCCTCGTTCTGATCGCTGCAACTACCAGAATCATTGCCCAAATGGCTCAGATGAAAAAAACTGCTTTTTTTGCCAACCAGGAAATTTCCATTGTAAAAACAATCGTTGTGTGTTTGAA  1440
Y  P  R  S  D  R  C  N  Y  Q  N  H  C  P  N  G  S  D  E  K  N  C  F  F  C  Q  P  G  N  F  H  C  K  N  N  R  C  V  F  E  466

AGTTGGGTGTGTGATTCTCAAGATGACTGTGGTGATGGCAGCGATGAAGAAAATTGCCCAGTAATCGTGCCTACAAGAGTCATCACTGCTGCCGTCATAGGGAGCCTCATCTGTGGCCTG  1560
S  W  V  C  D  S  Q  D  D  C  G  D  G  S  D  E  E  N  C  P  V  I  V  P  T  R  V  I  T  A  A  V  I  G  S  L  I  C  G  L  506

TTACTCGTCATAGCATTGGGATGTACTTGTAAGCTTTATTCTCTGAGAATGTTTGAAAGAAGATCATTTGAAACACAGTTGTCAAGAGTGGAAGCAGAATTGTTAAGAAGAGAAGCTCCT  1680
L  L  V  I  A  L  G  C  T  C  K  L  Y  S  L  R  M  F  E  R  R  S  F  E  T  Q  L  S  R  V  E  A  E  L  L  R  R  E  A  P  546

CCCTCGTATGGACAATTGATTGCTCAGGGTTTAATTCCACCAGTTGAAGATTTTCCTGTTTGTTCACCTAATCAGGCTTCTGTTTTGGAAAATCTGAGGCTAGCGGTACGATCTCAGCTT  1800
P  S  Y  G  Q  L  I  A  Q  G  L  I  P  P  V  E  D  F  P  V  C  S  P  N  Q  A  S  V  L  E  N  L  R  L  A  V  R  S  Q  L  586

GGATTTACTTCAGTCAGGCTTCCTATGGCAGGCAGATCAAGCAACATTTGGAACCGTATTTTTAATTTTGCAAGATCACGTCATTCTGGGTCATTGGCTTTGGTCTCAGCAGATGGAGAT  1920
G  F  T  S  V  R  L  P  M  A  G  R  S  S  N  I  W  N  R  I  F  N  F  A  R  S  R  H  S  G  S  L  A  L  V  S  A  D  G  D  626

GAGGTTGTCCCTAGTCAGAGTACCAGTAGAGAACCTGAGAGAAATCATACTCACAGAAGTTTGTTTTCCGTGGAGTCTGATGATACAGACACAGAAAATGAGAGAAGAGATATGGCAGGA  2040
E  V  V  P  S  Q  S  T  S  R  E  P  E  R  N  H  T  H  R  S  L  F  S  V  E  S  D  D  T  D  T  E  N  E  R  R  D  M  A  G  666

GCATCTGGTGGGGTTGCAGCTCCTTTGCCTCAAAAAGTCCCTCCCACAACGGCAGTGGAAGCGACAGTAGGAGCATGTGCAAGTTCCTCAACTCAGAGTACCCGAGGTGGTCATGCAGAT  2160
A  S  G  G  V  A  A  P  L  P  Q  K  V  P  P  T  T  A  V  E  A  T  V  G  A  C  A  S  S  S  T  Q  S  T  R  G  G  H  A  D  706

AATGGAAGGGATGTGACAAGTGTGGAACCCCCAAGTGTGAGTCCAGCACGTCACCAGCTTACAAGTGCACTCAGTCGTATGACTCAGGGGCTACGCTGGGTACGTTTTACATTAGGACGA  2280
N  G  R  D  V  T  S  V  E  P  P  S  V  S  P  A  R  H  Q  L  T  S  A  L  S  R  M  T  Q  G  L  R  W  V  R  F  T  L  G  R  746

TCAAGTTCCCTAAGTCAGAACCAGAGTCCTTTGAGACAACTTGATAATGGGGTAAGTGGAAGAGAAGATGATGATGATGTTGAAATGCTAATTCCAATTTCTGATGGATCTTCAGACTTT  2400
S  S  S  L  S  Q  N  Q  S  P  L  R  Q  L  D  N  G  V  S  G  R  E  D  D  D  D  V  E  M  L  I  P  I  S  D  G  S  S  D  F  786

GATGTGAATGACTGCTCCAGACCTCTTCTTGATCTTGCCTCAGATCAAGGACAAGGGCTTAGACAACCATATAATGCAACAAATCCTGGAGTAAGGCCAAGTAATCGAGATGGCCCCTGT  2520
D  V  N  D  C  S  R  P  L  L  D  L  A  S  D  Q  G  Q  G  L  R  Q  P  Y  N  A  T  N  P  G  V  R  P  S  N  R  D  G  P  C  826

GAGCGCTGTGGTATTGTCCACACTGCCCAGATACCAGACACTTGCTTAGAAGTAACACTGAAAAACGAAACGAGTGATGATGAGGCTTTGTTACTTTGTTAGGTACGAATCACATAAGGG  2640
E  R  C  G  I  V  H  T  A  Q  I  P  D  T  C  L  E  V  T  L  K  N  E  T  S  D  D  E  A  L  L  L  C                          859

AGATTGTATACAAGTTGGAGCAATATCCATTTATTATTTTGTAACTTTACAGTTAAACTAGTTTTAGTTTAAAAAGAAAAAATGCAGGGTGATTTCTTATTATTATATGTTAGCCTGCAT  2760

GGTTAAATTCGACAACTTGTAACTCTATGAACTTAGAGTTTACTATTTTAGCAGCTAAAAATGCATCACATATTGCATATTGTTCAATAATGGTCCTTTCATTTGTTTCTGATTGTTTTC  2880
                                                                                                                  ******

ATCCTGATACTGTAGTTCACTGTAGAAATGTGGCTGCTGAAACTCATTTGATTGTCATTTTTATCTATCCTATGTTAAATGGTTTGTTTTTACAAAATAATACCTTATTTTAATTGAAAC  3000
                                                                                                                  ******

GTTTATGCTTTTGCCAAGCACATCTTGTAACTTAATATAGCTAGATGTTAAGGTTGTTAATGTACCAAAAAAAAAAAA                                          3078
                                  ******
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Figure 3B

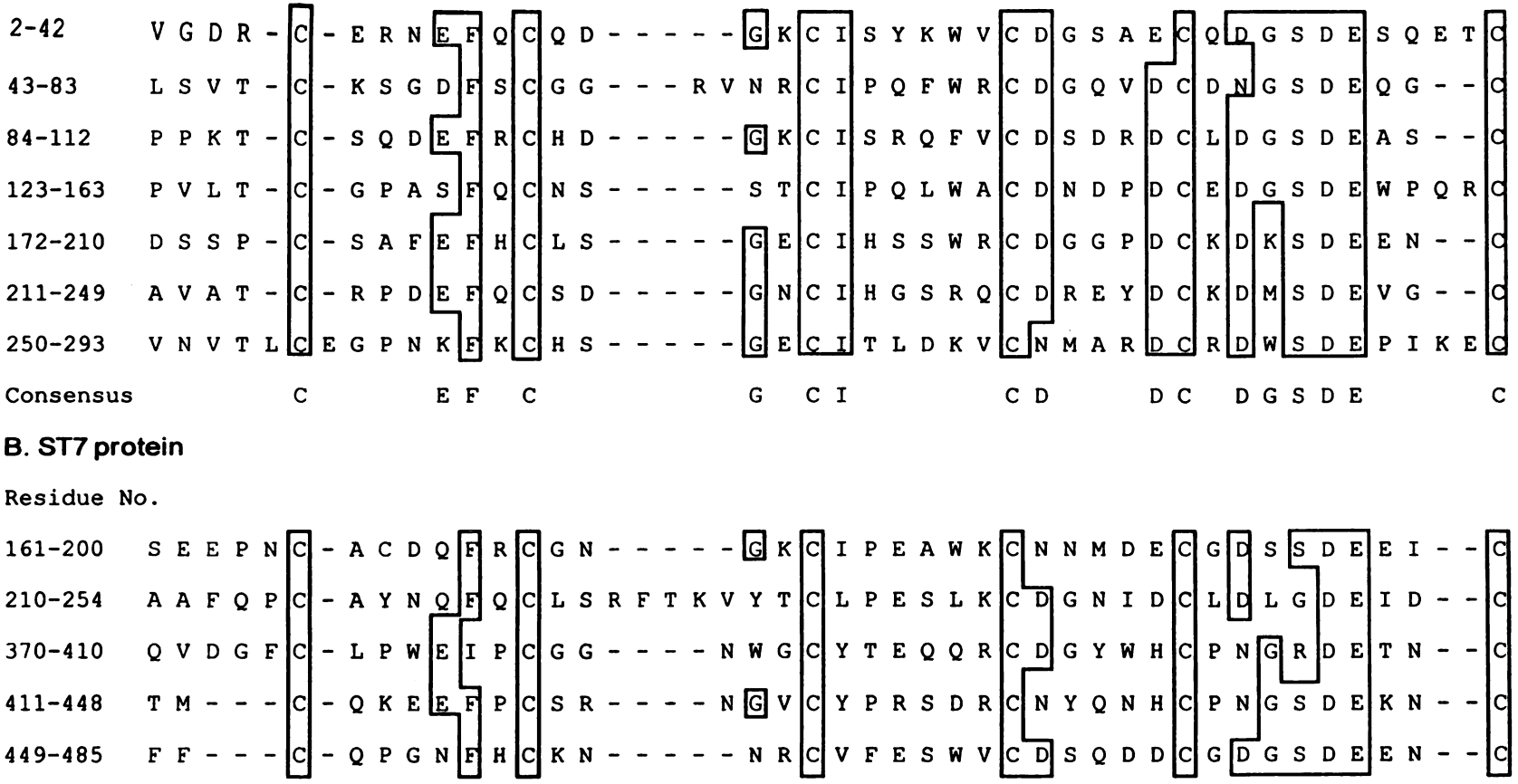
Pattern of Expression of ST7 mRNA in Various Human Tissues. Northern analysis using a human normal tissue blot (Clontech) revealed that ST7 cDNA hybridized to a single transcript of 3.7 kb. The ST7 mRNA was found to be most abundant in heart and skeletal muscle, expressed at moderate levels in brain, lung, placenta and pancreas, and barely detectable in tissues containing large number of epithelial cells such as liver and kidney (Figure 5).

Western Blotting Analysis of ST7 Protein in Human Tumor Cell Lines. A rabbit anti-ST7 polyclonal antibody (designated B250) was raised against a synthetic peptide as described in Materials and Methods. This antibody recognized a protein with an apparent molecular mass of 85 kDa, which is lower than the estimated molecular mass. The reason for this anomalous migration of ST7 protein in SDS-polyacrylamide gel is not clear. The specificity of this antibody for ST7 protein was tested by determining the extent of inhibition with the antigen peptide. After preincubation of the antibody with the antigen peptide (60 μM), the signal for the ST7 protein band in immunoblots was dramatically reduced (Figure 6). Using this antibody, the expression level of ST7 protein in normal cells and tumor-derived cell lines was analyzed by Western blotting analysis. As shown in Figure 7, MSU-1.1 cells and normal human fibroblast cell line SL89 exhibited high levels of ST7 protein, whereas the six malignant MSU-1.1 derivatives (lanes 3-8) and three cell lines derived from human fibrosarcomas (lanes 9-11) showed low levels of ST7 protein, which was consistent with the levels of ST7 mRNA transcripts in these cells (Figure 2). Interestingly, human fibrosarcoma-derived cell lines HT1080 and VIP:FT and rhabdomyosarcoma-derived cell line RD showed an mRNA level of ST7 comparable to that in MSU-1.1 cells (Figure 2, compare lane 9 and 11,14 and 15), whereas the protein was undetectable (Figure 7, compare lane 1 and 12-14). This result

Fig. 4. Alignment of the cysteine-rich repeat region of ST7 with that of the human LDL receptor. Amino acids are numbered on the left. Conserved amino acids are boxed. The data of human LDL receptor are taken with permission from "The LDL receptor gene: A mosaic of exons shared with different proteins" by Sudhof et al., Science, 1985.

A. LDL-receptor

Residue No.



B. ST7 protein

Residue No.

Fig. 4

suggests that downregulation of ST7 gene product occurs at both transcriptional and posttranscriptional levels.

Fig. 5. Expression of ST7 gene in normal human tissues. (A) Human multiple tissue blot (Clontech) containing poly $(A)^+$ RNA (2 μg/lane) from the indicated tissues probed with labeled 2.6 kb ST7 cDNA fragment. (B) The blot, stripped and rehybridized with a probe for β-actin as a loading control.

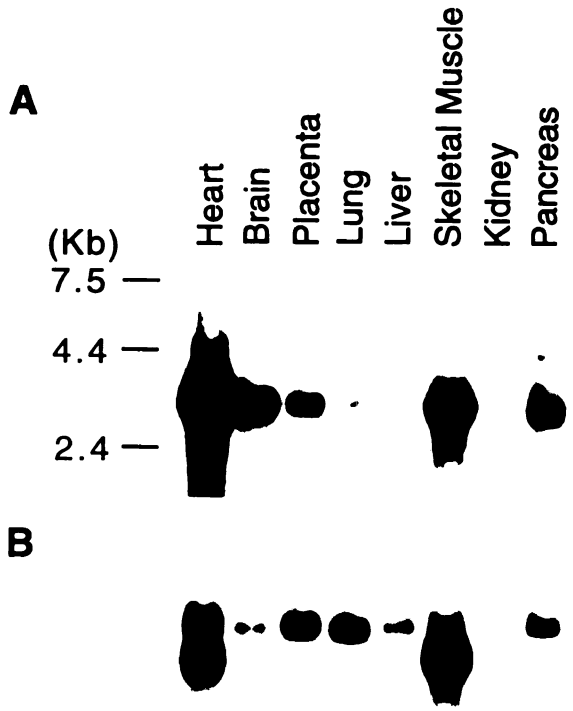

A
Heart
Brain
Placenta
Lung
Liver
Skeletal Muscle
Kidney
Pancreas
(Kb)
7.5
4.4
2.4
B

Fig.5

Fig. 6. Analysis of the specificity of the antibody B250. Immunoblots of cell lysate (25 μg protein/lane) from MSU-1.1 cells were incubated with the rabbit anti-ST7 peptide antibody B250 without (lane 1) or with (lane 2) prior incubation with the antigen peptide. The position of the ST7 signal is indicated by an arrow.

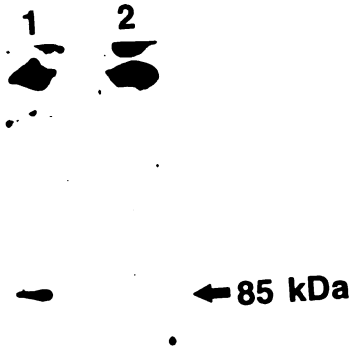


Fig. 6

Fig. 7. Western blot analysis of the expression of ST7 in normal human fibroblast cell lines (MSU-1.1 and SL89) and 12 tumor-derived cell lines. Cellular protein (25 μg) was analyzed by electrophoresis in 10% polyacrylamide gel and probed with antibody B250. The origin of the tumor-derived cell lines are: malignant cell lines derived from tumors formed in athymic mice after injection of MSU-1.1 cells transformed by various methods (lanes 3-8); malignant cell lines derived from a patient's fibrosarcoma (lanes 9-13) and rhabdomyosarcoma (lane 14).

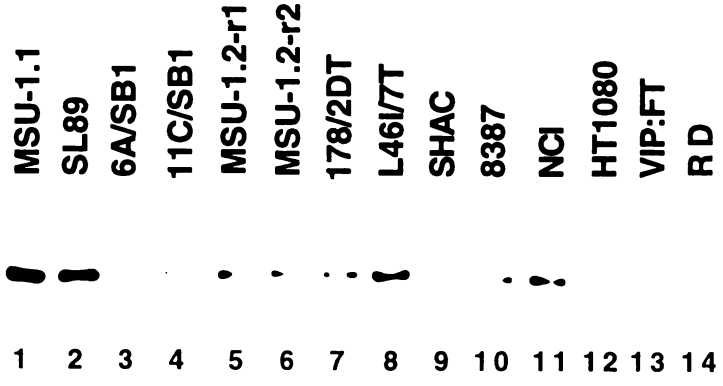

MSU-1.1
SL89
6A/SB1
11C/SB1
MSU-1.2-r1
MSU-1.2-r2
178/2DT
L46I/7T
SHAC
8387
NCI
HT1080
VIP:FT
R D
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 7

DISCUSSION

We have cloned and identified a novel gene, ST7, that encodes a 3.7 kb mRNA. Our analysis of ST7 expression in multiple human tumor-derived cell lines revealed that 10 out of 15 tumor-derived cell lines from patients exhibit low or no expression of ST7 mRNA. Without the normal cells from which these tumor cell lines were derived, we cannot be certain that the low expression resulted from downregulation. However, because we found that ST7 is widely expressed in normal human tissues, including heart, skeletal muscle, brain, lung, pancreas and placenta, but not in tissues consisting of a large number of epithelial cells, such as liver and kidney, and that a series of normal human fibroblast cell lines in culture expressed relatively constant levels of ST7 mRNA and protein (data not shown), we conclude that ST7 is ordinarily expressed at least in most cells of mesenchymal origin. Because six out of 16 tumor-derived cell lines from MSU-1.1 cells malignantly transformed by various methods clearly exhibited downregulation of ST7 mRNA and protein, we conclude that at least in mesenchymal-derived tumor cell lines, the downregulation of ST7 is frequently associated with neoplastic transformation.

Our data also suggest that the regulation of ST7 expression occurs at both the transcriptional and posttranscriptional levels. In most cases, the steady state protein level of ST7 correlates well with its mRNA level. However, in three malignant cell lines tested in this study, i.e., HT1080, VIP:FT and RD, which were derived from sarcomas taken from patients, the steady state mRNA level of ST7 was comparable to that seen in normal human fibroblasts, whereas the ST7 protein was barely detectable. This may reflect failure of translation initiation or a defect in the stability of the protein product.

According to the apparent molecular weight of the protein on SDS-PAGE gel, the cDNA we have isolated can account for the entire coding region of the novel protein. Of the two forms of the ST7 mRNA that we isolated, the shorter lacks 57 nucleotides at the 5' end and encodes a protein of 840 amino acids that lacks residues 27 to 45 found at the amino terminal end of the longer ST7 protein (indicated by double underline in Figure 3B). This omission does not affect the open reading frame. The two isoforms presumably result from alternative splicing. The significance of the 5' end heterogeneity awaits more detailed functional analyses.

The amino-terminal half of the predicted ST7 protein product contains five imperfect 40-amino acid repeats. This set of repeats is homologous to the cysteine-rich, ligand-binding domain in the human LDL receptor. Many cell surface and secreted proteins contain repeated cysteine-rich motifs that are each 40-50 amino acids in length and contain six cysteine residues linked in three disulfide bridges (Doolittle et al., 1984; Appella et al., 1988; Daly et al., 1995). The common functional characteristic of these repeats is their ability to mediate protein-protein interactions. Therefore it is possible that this conserved sequence in ST7 is involved in ligand binding. However, it cannot be ruled out that this cysteine-rich repeat sequence codes for a structural motif common to a number of extracytoplasmic protein domains. Since the ST7 protein shares no other significant homology with any known protein, we can only speculate as to its function. One possibility is that the ST7 protein is a component of a signal transduction pathway that negatively regulates cell growth. The predicted membrane-spanning structure of the ST7 protein suggests that it acts as a cellular receptor or co-receptor, functioning as a ligand-regulated suppressor of a signaling unit like the recently cloned tumor suppressor gene patched, which encodes a receptor for Sonic hedgehog (Stone et al., 1996).

Another possibility is that ST7 protein participates in cellular adhesion in a fashion similar to the candidate tumor suppressor gene DCC (for deleted in colorectal cancer) (Cho and Fearon, 1995; Fearon, 1996), or ST7 protein undergoes proteolysis on the cell surface, releasing a locally acting chemical signal. Any of these mechanisms could play a role in tumorigenesis. The cloning of the ST7 cDNA and the availability of the ST7-specific antibody should facilitate further experiments designed to better understand the function of the novel gene and its role in tumorigenesis.

MATERIALS AND METHODS

Cells and Cell Culture. The infinite life span human fibroblast cell strain MSU-1.1 and its derivative cell lines were routinely cultured in Eagle's minimum essential medium modified by addition of L-aspartic acid (0.2 mM), L-serine (0.2 mM) and pyruvate (1mM) and supplemented with 10% SCS (Hyclone Laboratory, Logan, UT), penicillin (100 units/ml), streptomycin (100 μg/ml) and hydrocortisone (1 μg/ml) (complete medium) at 37^0C in a humidified incubator containing 5% CO_2 in air.

Differential mRNA Display. Non-tumorigenic infinite life span human fibroblast cell strain MSU-1.1 and one of its tumorigenic derivative cell line, designated 6A/SB1, were used as sources of RNA for this study. Differential mRNA display and TA cloning were carried out essentially as described (Qing et al., 1997).

Northern Blot Analysis. Total RNA from cells in exponential growth was extracted using RNAzolB (Tel-Test, Friendswook,TX) according to manufacturer's instructions. For Northern blot analysis, RNA (15 μg) from each cell line was electrophoresed on 1.2% agarose/2.2 M formaldehyde gels, and then was transferred to hybond-N membrane and immobilized by UV crosslinking (UV Stratalinker 2400, Stratagene, La Jolla, CA). The cDNA probe was radiolabeled using a random primed labeling method (Feinberg and Vogelstein, 1983). The blots were hybridized as previously described (Qing et al., 1997). For analysis of ST7 expression in various normal human tissues, the Multiple Tissue Northern Blot was purchased from Clontech (Clontech, Palo Alto, CA). Variation in RNA loading per lane was evaluated by probing with the GAPDH cDNA or ß-actin as the controls.

Cloning of Human ST7 cDNA. The directional human fibroblast cDNA library (a generous gift from Dr. Legerski, The University of Texas, M.D. Anderson Cancer Center, Houston, that is referred to as Library 9) was used to obtain the full-length cDNA corresponding to the ST7 gene. We screened the library by the High Fidelity Expand PCR method (Boehringer Mannheim, Indianapolis, IN) with a vector-specific primer (5'-CCGGAAGCTTCTAGAGATCCCTCGA) and a ST7 gene specific primer based on the partial ST7 sequence obtained from differential display (5'-GCTCCAACTTGTATACAATCTCCC). Plasmid DNA derived from 10 x 10^6 independent clones was used as the template. The 50 μl PCR mixture contained 1.75 mM $MgCl_2$, 0.2 mM dNTP, 15 pmol of each primer, 100 ng of library plasmid DNA, and 2.5 units of the mixture Taq and Pwo DNA polymerase. The PCR cycling consisted of initial denaturation at 94^0C for 2 min, followed by 10 cycles of 94^0C for 30 s, 63^0C for 30 s, and 68^0C for 3 min, followed by another 20 cycles with the same parameters, except that the elongation time was extended for 15 s for each new cycle, followed by final elongation at 68^0C for 10 min. The PCR product was separated by electrophoresis in 1% agarose gel, and the major bands were purified using Qiaquick Gel Extraction kit (Qiagen, Chatsworth, CA). The purified DNA (about 2.6 kbp, noted as clone A) was used as probes for Northern analysis and cloned into the pCRII vector using the TA cloning method (Invitrogen, San Diego, CA).

To obtain additional 5' sequence of this gene, we screened a human skeletal muscle cDNA library (a generous gift from Dr. Ki-Han Kim, Purdue University, West Lafayette) by PCR using one primer from the 5' end of clone A, designated JM131 (5'-GGGTTGAAAAGCAGCAGGAGTTGGAGG) and another vector-specific primer from the region of the cloning site of λgt11 (5' GATTGGTGGCGACGACTCCTGGAGC). The

fragment generated was subcloned and designated clone B.

Clone C, which contains the first translation initiation codon, was isolated by the 5' rapid amplification of cDNA ends (5'-RACE) method using a human heart Marathon-ready cDNA (Clontech) with the ST7 gene-specific primer JM131. The PCR products were subcloned into the pCRII vector as above and sequenced.

DNA Sequencing and Sequence Analysis. Both strands of the cDNA inserts in the pCRII vector were sequenced manually by the dideoxy chain termination method with the SP6 and T7 primers using a Fidelity DNA Sequencing kit (Oncor, Gaithersburg, MD). For long cDNA inserts, synthetic oligonucleotides were used as primers to complete the sequence. Resolution was improved in some regions by replacing dGTP with deaza-dITP in the nucleotide mixture. The cDNA sequence and deduced protein sequences were analyzed by FASTA and BLAST program with the DNA and protein databases at the National Center for Biotechnology Information (NCBI). Secondary structure predictions and the properties of the putative protein were calculated using the GCG program (Genetic Computer Group, Madison, WI).

Production of Anti-ST7 Antibody. The peptide corresponding to the C-terminus of the ST7 protein (CLEVTLKNESTDDEA in the single-letter amino acid code; corresponding to amino acids 841 to 855 of ST7) was synthesized by the Macrostructural Facility of the Department of Biochemistry, Michigan State University. The synthetic peptide was coupled to keyhole limpet hemocyanin (KLH) with the chemical crosslinker glutaraldehyde. To obtain anti-ST7 antibody, 200 μg of KLH-conjugated peptide was emulsified with an equal volume of TiterMax (Cytrx, Norcross, GA) in a total volume of 1

ml, and 0.1 ml was injected subcutaneously into each of four sites on each of two female New Zealand White rabbits. The rabbits were administered booster shots after four weeks. They were bled on day 42 and 56 and serum was prepared according to standard protocol (Sambrook et al., 1989) and was designated B250.

Western Blotting Analysis. Cell lysates were prepared with RIPA buffer composed of 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and 0.15 units/ml aprotinin as described (Qing et al., 1997). Aliquots of cell lysates containing 25 μg of protein were mixed with the sample buffer (0.05M Tris-HCl, pH6.9, 9% glycerol, 2.3% SDS, 0.1% bromophenol blue and 5% β-Mercaptoethanol), separated on a SDS/polyacrylamide gel (10%), and electroblotted onto an Immobilon-P membrane (Millipore, Bedford, MA). The blots were incubated for 2 h at room temperature in Tris-buffered saline (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dry milk (blocking solution), and then incubated for 2 h at room temperature with B250, the rabbit anti-ST7 antibody, diluted 1:500 in the same solution. The blots were washed several times and then incubated with horseradish peroxidase-conjugated goat-anti-rabbit IgG (Sigma, St. Louis, MO) that had been diluted 1:5000 with blocking solution. Enhanced Chemiluminescence (Amersham, Arlington Heights, IL) was used according to the manufacturer's recommendations to detect the signal.

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