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Oncogenes and Tumor Suppressor Genes in Human Malignancies

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Dedication

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(1937–1992)

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Series Editor
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We dedicate this volume to the memory and achievements (both scientific and academic) of one of the most influential cancer researchers of this century, particularly relating to the biology and treatment of breast cancer. He will be greatly missed both personally and professionally.

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Preface

The first edition of *Oncogenes* (1989) focused on several of the better known transforming mechanisms and surveyed a spectrum of solid tumors and hematologic malignancies. Several of the nearly 50 known oncogenes most relevant to human disease were examined. In contrast, this volume presents a very different profile and balance of subject material that reflects the rapidly changing field of molecular oncology and its newly emerging concepts. Among the most important discoveries of the past 4 years are the identification of nearly a dozen different tumor suppressor genes and the finding of an entirely new class of cancer-causing gene (*bcl*-2) that acts by inhibiting cell death rather than stimulating cell proliferation.

This edition begins by reviewing selected malignancies in which our earlier search for clinically relevant oncogenes has led to more focused studies on gain-of-function and loss-of-function genetic abnormalities, as well as autocrine and paracrine growth factor loops known to regulate tumor physiology and malignant cell behavior. Curiously, many of these genetic and functional abnormalities are shared by several different tumor types and are not uniformly present in all tumors of the same type. This observation brings up molecular questions about the tissue-specific determinants that underlie individual cancers and also gives added impetus to the suggestion that molecular abnormalities (referred to as tumor markers) be included among the histopathologic features used for clinical diagnosis and management. The remainder of the volume updates molecular mechanisms relating to selected growth factor systems, oncogenes, and tumor suppressor genes introduced earlier in the disease-oriented chapters. The topics are not meant to be inclusive or to assign undue importance to any particular oncogenic mechanism; rather, the selections serve to highlight the concept that growth factors often mediate nonproliferative processes, such as tumor cell invasion and metastasis, two critical functions that distinguish the behavior of cancers from benign human tumors.

Together, these reviews try to convey the increasing fascination that comes with a greater understanding of tumor physiology. They reveal, for instance, that tumor cells can induce the secretion of paracrine growth factors from nonmalignant tissues, thus usurping normal genetic programs

reserved for fetal development or wound healing, such as those that provide neovascularization for a malignant tissue colony. Optimistically, the uncovering of these subverted intercellular mechanisms, as well as the constitutively stimulated intracellular signaling pathways associated with activated oncogenes and mutated tumor suppressor genes, provide researchers with many new targets for the development of more specific and less toxic anticancer agents. As pointed out in the following chapters, a number of these novel agents are already in clinical testing.

CHRISTOPHER C. BENZ
EDISON T. LIU

Oncogenes and Tumor Suppressor Genes in Human Malignancies

1. Oncogenes and tumor suppressor genes

Edison Liu and Bernard Weissman

Introduction

Cancer-associated genes generally can be divided into 'dominant' acting protooncogenes/oncogenes and 'recessive' tumor suppressor genes. Most simply defined, protooncogenes are identified by a gain of function as mutational damage occurs, whereas tumor suppressor genes contribute to cancer by a loss of function. However, the pace of science leads us to believe that these categories are arbitrary and, perhaps, functionally incorrect as the oncogenes are found to interact with tumor suppressor genes. Nevertheless, because the details of the interaction are, to date, still unclear, the current nosology of oncogenes and tumor suppressor genes will be used for the sake of convenience. The ever increasing numbers of proto-oncogenes and tumor suppressor genes discovered and their complex interactions suggest that the challenge for cancer researchers lies in deciphering the intricate genetic mosaic that characterizes cancer. This chapter will serve as an overview for both types of cancer-associated genes and will include specific examples that highlight important concepts in the field.

Oncogenes

Viral oncogenes were the first proof that endogenous genes can directly cause cancer. Here, the mutations are engendered when normal cellular genes (proto-oncogenes, designated by the c- prefix) are mutated through the error-prone replicative process of the retroviral life cycle. The end result is a viral oncogene (v-*onc*) that structurally resembles its normal cellular counterpart but is functionally locked in a biochemically activated form. Since cancer cells differ from normal cells mainly by the loss of normal control mechanisms for cell growth and differentiation, it is not surprising that all proto-oncogenes identified thus far are involved in different aspects of these command and control processes.

Cells are triggered to change and grow by external signals; therefore, proto-oncogenes and oncogenes figure heavily in signal transduction, a

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process that converts external stimuli into intracellular signals that guide cellular function. In the past 10–15 years of oncogene research, the identification of proto-oncogenes as specific components of signal transduction pathways has been the major discovery in the field. These and other more recent findings suggest that several new areas are emerging as important topics for future investigations in molecular oncogenesis. They include the deciphering of the intricate interactions between the various oncogene classes involved in signal transduction, the study of the molecular mimicry produced by chimeric transcription factors, and the recognition that oncogenesis may result from a block to programmed senescence.

Interactions between oncogene pathways

Proto-oncogenes interact with each other in different cellular functions with astonishing consistency, suggesting that an understanding of these oncogene networks may lead to a better grasp of the biochemical controls of tumorigenesis. Intracellular injection of anti-*ras* neutralizing antibodies revealed that transformation by certain membrane-associated oncogenes, such as *src*, *fms*, and *fes*, were blocked by *ras* inactivation, but that transformation by two cytoplasmic oncogenes *mos* and *raf* was not affected [1]. These data and the finding that *ras* and *myc* are both required to transform primary rodent fibroblasts were the first indications that oncogenes interact to transform cells [2].

Since then, many nodes of interaction amongst the proto-oncogenes have been discovered and with some intriguing surprises [reviewed in 3]. The platelet-derived growth factor (PDGF) and its oncogenic homologue, *v-sis*, function as a hormone; its receptor (PDGF-receptor) is a receptor tyrosine kinase, of which c-*erbB* (a.k.a, epidermal growth factor receptor), HER-2/*neu*, and c-*fms* (a.k.a, the CSF1 receptor) are also members. These receptor tyrosine kinases act to transduce external stimuli into intracellular signals via tyrosine phosphorylation. Upon stimulation, the activated receptor not only phosphorylates a variety of substrates (including itself) but also functions as a potential point of aggregation for critical cellular components. One target for this enzymatic activity and topographical recruitment is the *ras*-GTPase activating protein (GAP), which undergoes association and phosphorylation with PDGFR after ligand activation [4,5]. GAP is the important cofactor that stimulates the *ras* GTPase activity responsible for converting the active *ras* form (*ras*-GTP) to the inactive *ras*-GDP form. *ras*-GTP complexes, stabilized either by nonhydrolyzable GTP analogs or by oncogenic mutations in *ras*, function to signal a variety of cellular responses, including proliferation and differentiation [6]. There is also evidence that at least in one system, *ras*-GTP must interact with GAP for *ras* action to be manifest [7]. Exactly how GAP is involved in PDGF-receptor signalling is unclear, but these data suggest that GAP may function as one such node of oncogene interaction.

Also converging on this node centered on the ligand-activated PDGFR is the c-*raf* protein product that coimmunoprecipitates with and is tyrosine phosphorylated by PDGFR [8]. The phosphorylated form of *raf* activates the serine/threonine kinase activity of the *raf*-1 protein product, which in turn induces (by yet undefined mechanisms) the transcription of the nuclear protooncogene c-*fos* [9]. Other regulatory proteins, such as phosphatidylinositol 3-kinase (PtdIns 3-kinase) and PLC-γ also undergo ligand-dependent association with and phosphorylation by the PDGFR; nevertheless, the association of several protooncogenes (PDGFR → GAP and c-*raf* → c-*fos*) in a single signal transduction pathway suggests that the oncogenic conversion of one gene may act in part by stimulating downstream protooncogenes. These biochemical links also suggest that pharmacologic intervention at a critical node of interaction may be devised to block transformation by a series of upstream oncogenes. Thus, for example, dominant inhibitory mutants or antisense constructs of *raf*-1 can attenuate *ras*-induced transformation [10].

Though *ras* appears to play a central role in a variety of signalling pathways, the linkage appears to be frequently through *ras*-associated proteins, such as GAP. In human disease this GAP-*ras* interaction figures prominently in neurofibromatosis I. The neurofibromatosis gene (*NF1*), localized on chromosome 17q11.2, when sequenced, has been found to be highly homologous to human GAP. Functional analysis confirmed that the normal *NF1* gene product acts to stimulate *ras*-p21 GTPase activity (a GAP activity) and complements the yeast GAP homolog, IRA2. Furthermore, *NF1* binds with greater avidity to *ras* than *ras*-GAP itself, though its specific activity is significantly lower [11,12]. These findings raise the intriguing possibility that NF1 functions to downregulate the active *ras*-GTP form and that mutations in *NF1*, like those found in neurofibromatosis, may inactivate its potentially important *ras*-suppressing effect.

These are but a few examples of the rich intracellular network involving the various oncogenes that can start to explain the nuances in signal transduction and the complexities of cellular transformation. We anticipate that further dissection of this network will lead us to a more unified theory of growth, differentiation, and transformation.

Molecular mimicry: Chimeric transcription factors

Whereas transformation by receptor tyrosine kinases can be the result of an excess of common signals, transformation by certain transcription factors arises by misdirecting signals. An example of this is seen in the genes involved in acute promyelocytic leukemia (APL). It has been long known that APL is characterized by a consistent cytogenetic translocation t(15;17)(q21;q11.2–12) [13]. Recently, using positional cloning techniques, the precise breakpoint has been mapped to the retinoic acid receptor alpha (RARA) gene on chromosome 17q [14,15]. The translocation results in a

fusion between a large 3′ portion of the RARA gene with the 5′ portion of a gene on chromosome 15 called *PML*.

PML is a putative zinc finger protein and a potential transcription factor resembling other human transcription factors such as the human recombinase-activating gene, *RAG*-1, and the repair gene *RAD*-18 of *Saccharomyces cerevisiae*. The fusion protein places the presumed DNA binding domain of *PML* with the ligand and DNA binding regions of *RARA* [16]. In CAT assays using target constructs harboring various retinoic acid response elements, the PML-RARA fusion protein was shown to function as a transcriptional activator. However, the PML-RARA protein exhibited a different transactivational profile compared to the native RARA protein [17]. This suggests that the PML-RARA fusion protein misdirects the retinoic acid signal to inappropriate DNA targets, a possible example of molecular mimicry.

The excitement of these findings were compounded by the fact that, clinically, the retinoic acid isomers, *cis*- and *trans*-retinoic acid, have been shown to induce differentiation in APL cells and complete remissions in APL cases [18,19]. Thus the molecular lesion in APL (i.e., in the retinoic acid receptor) makes conceptual sense with the known clinical information (i.e., response to retinoic acid derivatives). Exactly how retinoic acid induces differentiation in leukemic cells harboring the PML-RARA fusion protein is unclear; the most simple explanation may be that PML-RARA functions as an oncogene that blocks the differentiation of myeloblasts at the promyelocyte stage. Treatment by retinoic acid may work by binding the fusion receptor, shunting the ligand–receptor complex to other sites, and relieving the block in differentiation.

This concept has precedence in a related oncogene, v-*erb*A, which contributes to avian erythroleukemias by blocking the normal cellular counterpart, the thryoid hormone receptor [20]. Structurally, RARA and c-*erb*A/thyroid hormone receptor (TR) are related. Now, evidence ties c-*erb*A/TR and RARs together biochemically in that a cell factor, the retinoic acid X receptor β or RXRβ, forms heterodimers with TRs and RARs, and enhances the binding of both RARs and TRs to their respective response elements [21]. This suggests that the RXRs may function as a node of interaction between two differentiation systems (TRs and RARs) shown to be operative in leukemogenesis. Again, the seemingly disparate oncogenic systems appear to coalesce to produce a more unified model of transformation.

This molecular mimicry has also been found in other human cancers. Pre-B lymphoblastic leukemias with the t(1;19) translocation result in fusion between the transactivation region of the E2A transcription factor and the DNA binding domain of the homeoprotein PBX [22]. Theoretically, in this fusion the E2A transcriptional activation is misdirected by the PBX DNA binding domain.

Blocking programmed senescence: bcl-*2*

Until recently the study of molecular oncogenesis has concentrated on growth and growth signals. On a kinetic level, however, the accumulation of cancer cells can be accomplished by a decrease in cell loss as well as by increased cellular proliferation. Current evidence suggests that the abrogation of programmed cell death or apoptosis may be an important concomitant to neoplastic transformation. The clearest example of an oncogene involved in the apoptotic process is *bcl*-2.

Follicular lymphomas are characterized by a consistent translocation between chromosomes 14 and 18 [t(14;18(q32;q21))]. The breakpoint on chromosome 14 involves the IgH locus, and that on chromosome 18 involves a novel oncogene, *bcl*-2 [23]. The novelty of *bcl*-2 was that the mechanism of transformation by *bcl*-2 was initially a mystery. The translocation did not perturb the structure of the oncoprotein but juxtaposed the IgH enhancer with the translocated *bcl*-2 gene [24]. This suggested that overexpression or inappropriate expression of *bcl*-2 in lymphoid cells leads to lymphomagenesis. However, standard transformation assays in 3T3 cells did not show any oncogenic activity with constructs overexpressing *bcl*-2. When exogenous *bcl*-2 was expressed in primary bone marrow cells through retroviral gene transfer or in transgenic mice, no early transformation was seen, though polyclonal lymphocytes with a capacity for prolonged survival in vitro dominated the transgenic lymph nodes [25].

The first indication that *bcl*-2 was involved in a transformation pathway that differed from other oncogenes was when *bcl*-2 was introduced into IL-3-dependent lymphoid and myeloid cell lines. These cell lines require IL-3 for growth in culture and will undergo apoptotic death when IL-3 is withdrawn. Other oncogenes, such as v-*fms* or v-*abl*, will induce factor-independent growth in these cell lines (FDC-P1 and LyH7). *bcl*-2, however, does not induce growth but, intriguingly, prevents FDC-P1 and LyH7 from undergoing programmed cell death when IL-3 is withdrawn [26,27].

That *bcl*-2 is directly involved in apoptosis in vivo has been confirmed by several lines of experimentation. *bcl*-2 expression appears to normally rescue antigen-reactive B cells as part of the process of antibody affinity maturation [28]. Immunohistochemical examination of normal lymphoid tissues show that the *bcl*-2 protein is most abundantly expressed in the long-lived recirculating B cells of the follicular mantle [29] and not in centroblasts and centrocytes that are destined to die. Lastly, the ability of the Epstein-Barr virus (EBV) to immortalize B cells may be due to its capacity to induce *bcl*-2 expression [30]. Thus *bcl*-2 represents an oncogene with a novel function: not the stimulation of proliferation, but the prevention of cell death.

Though *bcl*-2 does not immediately transform cells, it contributes significantly to malignant conversion. Transgenic mice harboring *bcl*-2 will

develop diffuse large cell lymphomas after long latency, and half of these tumors will harbor rearrangements of the *myc* oncogene [31]. This interaction between *myc* and *bcl*-2 have also been confirmed by the inevitable emergence of early lymphomas in the progeny of matings between *myc*- and *bcl*-2-bearing transgenic mice [32].

Tumor suppressor genes

Cancer behaves as a recessive genetic trait

In the early 1960s, a French group led by Drs. Barski and Cornefurt observed the phenomenon of fusion of cultured mouse cells grown in the laboratory [33]. Their studies showed that a 'hybrid' cell could arise that contained all of the genetic information of two different cells. After the development of efficient methods for this process, investigators could easily produce hybrid cells from any two cell types. Using this technology, several investigators produced hybrid cells between mouse or human cancer cells and normal fibroblastic cells of the same species. They then determined whether these hybrid cells had retained the malignant phenotype of the cancer cell. Remarkably, all of the hybrid cell lines had lost the ability to form tumors in vivo [34]. Thus, the results established the recessive genetic nature of malignancy. Furthermore, these experiments suggested that normal cells contain information that can modulate or abrogate the malignant nature of cancer cells.

Retinoblastoma, the simple model for the development of human cancer

The occurrence of retinoblastoma in children was one of the first cancers with a demonstrated familial inheritance. The tumor appears sporadically at a rate of approximately 1 in every 10,000 live births. However, children of individuals with the hereditary form of this disease have a 50-50 chance of developing retinoblastoma. Initially, this disease was classified as a dominantly inherited trait, in a similar manner to Huntington's disease or Marfan's syndrome. However, in 1970 Dr. Alfred Knudson proposed that retinoblastoma actually arose from a loss of genetic information, rather than a single dominantly acting gene, such as an oncogene [35]. The basis for this assertion lay in a statistical analysis of two facets of the disease. In simplest terms, the majority of the patients who had the bilateral disease (involvement of both eyes) also possessed the hereditary form, while none of patients with nonhereditary retinoblastoma showed bilateral disease. Furthermore, patients with the hereditary disease tended to develop tumors at an earlier age than individuals with the other form. Dr. Knudson hypothesized that retinoblasts must lose both copies of a recessively acting or tumor-suppressor gene in order for retinoblastoma to occur. In non-

hereditary or sporadic cases, two separate genetic events must occur for loss of both copies of the gene, a relatively rare occurrence. However, in the hereditary disease individuals would inherit one abnormal copy of this gene from the affected parent. Thus, only a single mutation or deletion of the remaining active gene would lead to the onset of this cancer.

From this initial observation in the early 1970s, a series of scientific clues from many laboratories led to the isolation of the retinoblastoma susceptibility gene (*rb*-1) in 1986. We will mention the highlights of this search because they illustrate the elegance and excitement of a scientific venture using the latest and best experimental tools. The first major piece of information coincided with the development of chromosome banding techniques that accurately identified each chromosome in human cells. A cytogenetic study of retinoblastoma tumors revealed a specific deletion in a small area of human chromosome 13 in about 25% of the cases [36]. The information lost in this area of the chromosome presumably contained the putative retinoblastoma susceptibility gene. Further support for this hypothesis came from the observation that the gene that codes for esterase D also localized to this region of chromosome 13 [37]. A case study of one patient with hereditary retinoblastoma showed that his somatic cells possessed only 50% of the normal esterase D [38]. However, the tumor that arose in this patient had lost all esterase D activity. The investigators interpreted these results as demonstration of a close physical linkage between the esterase D and the then putative *rb*-1 genes. This individual had inherited a deletion of chromosome 13, including both the esterase D and *rb*-1 genes from his affected parent. Subsequent loss of the remaining normal *rb*-1 gene and its closely linked esterase D gene led to the development of retinoblastoma.

Even with these results, a major problem remained with the proposed location of the *rb*-1 gene on chromosome 13. As mentioned earlier, only about 20–25% of the tumors showed visible deletions in chromosome 13. Even in these cases, generally only one chromosome 13 possessed a detectable deletion. Therefore, the majority of the retinoblastoma tumors displayed two apparently normal human chromosome 13s. How could one reconcile the required loss of genetic information on the chromosome 13 with the seeming apparently normal chromosomes in the tumors? The answer to this conundrum came with the development of new molecular markers, called restriction fragment length polymorphisms (RFLP). These DNA sequences allowed investigators to distinguish between the two copies of each human chromosome pair. When scientists compared these RFLP markers between tumor and normal material from patients with retinoblastoma, they observed an exciting result. While normal cells possessed two different copies of chromosome 13, presumably representing the contribution of both parents, tumor material contained two copies of the same chromosome [39]. Thus, during tumor progression the cancer cells had lost one complete copy of chromosome 13 and had replaced it with a duplicate

copy of the remaining chromosome 13. If this remaining copy of the chromosome carried an inactive *rb*-1 gene, the retinoblastoma cell now had two inactive copies. Subsequent studies showed that this scenario occurs frequently during the genesis of retinoblastoma.

By determining the frequency of loss of the molecular markers in multiple tumor samples, investigators found several that lay close to the *rb*-1 gene. Using these random sequences of DNA as starting points, Dr. Dryja's and Dr. Weinberg's laboratories isolated the *rb*-1 gene based on its position on chromosome 13 [40]. Further studies showed that this candidate *rb*-1 gene was lost or mutated in all retinoblastoma cells [41].

Functions of tumor suppressor genes

What role does the *rb*-1 protein play in the normal function of cells? The previous section discussed the involvement of proto-oncogenes in the control of cellular proliferation. These proteins provide positive signals for the onset and maintainence of cell growth. The *rb*-1 gene exerts a negative effect on the cell cycle, i.e., it applies the 'brake' to the positive signals of the oncogenes. One of the first clues to *rb*-1 function came from the field of DNA tumor viruses. Several investigators had noted that the transforming genes of these viruses bound to a variety of cellular host proteins. One of these previously unidentified proteins had a molecular weight of approximately 107 kDa, the same size as the *rb*-1 protein. Later, using specific antisera, this *rb*-1 protein was found to be the same 107-kDa protein that complexed with the viral transforming proteins SV40 large T antigen and the adenovirus E1A protein [42,43]. Thus, these viral transforming proteins inactivate *rb*-1 function by sequestering *rb*-1 in an inactive complex. Normally, the *rb*-1 protein negatively regulates an important transcription factor, E2F. This sequestration of the *rb*-1 gene product presumably releases this suppression of E2F, thus allowing promiscuous positive growth signalling [44,45]. Therefore, one can see how the loss of this gene in the retinoblasts may lead to uncontrolled cell division, a hallmark of the cancer cell. Experimentally, restoration of a normal *rb*-1 gene to retinoblastoma cells causes a potent inhibition of cell growth [46].

Another feature of oncogenes concerns their involvement in different types of human cancers. Thus, activation of the *ras* family or amplification of the *neu* oncogene may occur in many types of carcinomas. In a similar fashion, loss of *rb*-1 function arises in a variety of human cancers. One study demonstrated a dramatic increase in cancer during young adulthood for patients who had survived hereditary retinoblastoma as a child [47]. These individuals displayed a restricted range of malignancies, including (in order of frequency) osteosarcomas, fibrosarcomas, and skin carcinomas. Examination of tumor material from these patients confirmed the inactivation of the *rb*-1 gene. Furthermore, many sporadic cases of these diseases also

contain losses of *rb*-1 function. This involvement of the *rb*-1 gene in the genesis of multiple human cancers corresponds to the similar patterns observed with oncogene activation.

The identification of tumor-suppressor genes has lagged behind the studies on oncogenes due to the paucity of appropriate assays. However, several research groups over the past 6 years have isolated approximately 10 tumor-suppressor genes. At least one of them, the p53 gene, functions as a regulator of the cell cycle [48]. Recently, Malkin et al. have demonstrated a loss of p53 activity in patients with the heritable Li-Fraumeni multicancer syndrome [49]. Interestingly, osteosarcoma, a tumor that loses *rb*-1 function, also shows deletion of the p53 gene. This seeming requirement for loss of two genes might explain the later appearance in childhood of osteoscarcoma compared to retinoblastoma. The development of the latter disease may result from the inactivation of a single tumor suppressor, *rb*-1, while the progression of osteoscarcoma may necessitate the loss of both genes.

Tumor suppressor genes and oncogenes — Opposite sides of the same coin?

The actions of oncogenes and tumor-suppressor genes intersect at other points in the normal pathways of cell growth. A candidate gene has been identified that is responsible for the onset of neurofibromatosis, a disease of neural crest cells [50,51]. The gene, NF1, has been discussed previously and codes for a large 250-kDa protein that contains a region similar to the GTPase activating protein (GAP) [52]. The GAP protein interacts with the *ras* gene family by stimulating the conversion of GTP to GDP by the *ras* p21 proteins [53]. The amount of p21 proteins bound to GTP correlates with an increase in cellular proliferation. Thus loss of the NF1 gene could potentially cause an increase in cell division by allowing an abundance of the active GTP-p21 protein complex.

Wilms' tumor, a pediatric nephroblastoma, may also present in hereditary or spontaneous forms with unilateral or bilateral involvement in a similar fashion to retinoblastoma. Two laboratories simultaneously isolated one of the genes important for the development of this disease, called *WT*-1 [54]. Unlike the *rb*-1 or p53 genes, only a limited number of tissue types produce the *WT*-1 protein, including kidney and thymus. The *WT*-1 gene codes for a transcription factor similar to the PML gene, a protein that regulates activity of other genes. However, *WT*-1 exerts a negative effect on the transcription of other cellular genes. Two of the proteins that *WT*-1 controls are growth factors. Thus, loss of *WT*-1 synthesis leads to abnormally high levels of proteins that stimulate proliferation. If this event occurs during fetal development, the overgrowth of these cells could provide the environment for the appearance of cancer cells.

Up to now we have viewed cancer as the product of single genetic events in different tumor types. However, human malignancies, especially adult carcinomas, arise from a series of oncogene perturbations and losses of tumor suppressor genes. The best example of this complex pathway for converting normal cells to malignant cells centers on recent studies on the genesis of colorectal cancer. The appearance of this tumor involves at least four different genetic alteractions, including loss of the p53 gene and activation of the K-*ras* oncogene. In the past 2 years several groups have characterized the role of two other tumor suppressor genes, DCC and APC, in the progression of colorectal cancer [55,56]. We must emphasize that the progression of this disease does not result from a defined temporal order of these genetic changes. Rather, the critical facet of this disease centers on the accumulation of these changes. Thus, all cases of advanced colorectal cancer show multiple genetic alterations, though the array of changes varies among individual tumors. In view of the complex nature of cellular growth with multiple redundant control mechanisms, it is not surprising that malignancy requires alterations in many genetic systems before the cancerous phenotype is manifest.

References

1. Smith MR, DeGudicibus SJ, Stacey DW: Requirement for c-ras proteins during viral oncogene transformation. Nature 320:540–543, 1986.
2. Land H, Parada LF, Weinberg RA: Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304:596–606, 1983.
3. Cantley LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R, Soltloff S: Oncogenes and signal transduction. Cell 64:281–302, 1991.
4. Kaplan DR, Morrison DK, Wong G, McCormick F, Williams LT: PDGF b-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex. Cell 61:125–133, 1990.
5. Kazlauskas A, Ellis C, Pawson T, Cooper JA: Binding of GAP to activated PDGF receptors. Science 247:1578–1581, 1990.
6. McCormick F: ras GTPase activating protein: Signal transmitter and signal terminator. Cell 56:5–8, 1989.
7. Yatani A, Okabe K, Polakis P, Halenbeck R, McCormick F, Brown AM: ras p21 and GAP inhibit coupling of muscarinic receptors to atrial K^+ channels. Cell 61:769–776, 1990.
8. Morrison DK, Kaplan DR, Escobedo JA, Rapp U, Roberts TM, Williams LT: Direct activation of the serine/threonine kinase activity of raf-1 through tyrosine phosphorylation by the PDGF-β receptor. Cell 58:649–657, 1989.
9. Jamal S, Ziff E: Transactivation of c-fos and beta-actin genes by raf as a step in early response to transmembrane signals. Nature 344:463–466, 1990.
10. Kolch W, Heidecker G, Lloyd P, Rapp UR: Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. Nature 349:426–428, 1991.
11. Xu Gangfeng, Lin Boris, Tanaka B, Dundd Diane, Wood D, Gesteland R, White R, Weiss R, Tamano F: The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of *S. cerevisiae*. Cell 63:835–841, 1990.

12. Martin GA, Viskochil D, Bollag GA, McCabe PC, Crosier WJ, Haubruck H, Controy L, Clark R, O'Connell P, Cawthon RM, Innis MA, McCormick F: The Gap related domain of the neurofibromatosis type 1 gene product interacts with ras p21. Cell 63:843–849, 1990.
13. De Braekeleer M, Lin CC: The occurrence of the 15;17 translocation in acute promyelocytic leukemia. Cancer Genet Cytogenet 19:311–319, 1986.
14. H de The, Chomienne C, Lanotte M, Degos L, Dejean A: The t(15;17) translocation of acute promyelocytic leukemia fuses the retinoic acid receptor a gene to a novel transcribed locus. Nature 347:558–561, 1990.
15. Borrow J, Goddard AD, Sheer D, Solomon E: Molecular analysis of acute promyelocytic leukemia breakpoint cluster region on chromosome 17. Science 249:1577–1580, 1990.
16. Goddard AD, Borrow J, Freemont PS, Solomon E: Characterization of a zinc finger gene disrupted by the t(15;17) in acute promyelocytic leukemia. Science 254:1371–1374, 1991.
17. Kakizuka A, Miller WH, Umesono K, Warrell RP, Frankel SR, Murty VVVA, Dmitrovsky E, Evans RM: Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RARA with a novel putative transcription factor, PML. Cell 66:663–674, 1991.
18. Castaigne S, Chomienne C, Daniel MT, Ballerini P, Berger R, Fenaux P, Degos L: All trans-retinoic acid as a differentiation therapy for acute promyelocytic leukemia: I. Clinical results. Blood 76:1704–1709, 1990.
19. Huang ME, Yu-Chen Y, Shu-rong C, Jin-Ren C, Jia-Xiang L, Long-Jun G, Zhen-Yi W: Use of all trans retinoic acid in the treatment of acute promyelocytic leukemia. Blood 72:567–572, 1988.
20. Zenke M, Munoz A, Sap J, Vennstrom B, Beug H: v-erbA oncogene activation entails the loss of hormone dependent regulator activity of c-erbA. Cell 61:1035–1049, 1990.
21. Leid M, Kastner P, Lyons R, Nakshatri H, Saunders M, Zacharewski T, Chen JY, Staub A, Garnier JM, Mader S, Chambon P: Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell 68:377–395, 1992.
22. Kamps MP, Murre C, Sun X, Baltimore D: A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B ALL. Cell 60:547–555, 1990.
23. Haluska FG, Finver S, Tsujimoto Y. Croce CM: The t(14;18) chromosomal translocations onvolved in B cell neoplasms result from mistakes in VDJ joining. Nature 324:158–161, 1986.
24. Seto M, Jaeger U, Hockett RD, Graninger W, Bennett S, Goldman P, Korsmeyer SJ: Alternative promoters and exons, somatic mutation and deregulation of the bcl-2-Ig fusion gene in lymphoma. EMBO J 7:123–131, 1988.
25. McDonnell TJ, Deane N, Platt FM, Nunez G, Jaeger U, McKeam JP, Korsmeyer SJ: bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell 57:79–88, 1989.
26. Hockenberry D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ: bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348:334–336, 1990.
27. Nunez G, London L, Hockenberry D, Alexander M, McKearn JP, Korsmeyer SJ: Deregulated bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. J Immunol 144:3602–3610, 1990.
28. Liu YJ, Joshua DE, Williams GT, Smith CA, Gordon J, MacLennan ICM: Mechanism of antigen-driven selection in germinal centres. Nature 342:929–931, 1989.
29. Pezzella F, Tse AGD, Cordell JL, Pulford KAF, Gatter KC, Mason DY: Expression of the bcl-2 oncogene protein is not specific for the 14;18 chromosomal translocation. Am J Pathol 137:225–232, 1990.
30. Henderson S, Rowe M, Gregory C, Croom-Carter D, Wang F, Longnecker R, Kieff E, Rickinson A: Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell 65:1107–1115, 1991.
31. McDonnell TJ, Korsmeyer SJ: Progression from lymphoid hyperplasia to high-grade

malignant lymphmoa in mice transgenic for the t(14;18). Nature 349:254–256, 1991.
32. Strasse A, Harris AW, Bath ML, Cory S: Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature 348:331–333, 1990.
33. Barski G, Sorieul S, Cornefurt FR: Production dans des coltures in vitro de deux souches cellulaires en association de cellules de caractere 'hybride.' Compt Rend Acad Sc 251L: 1825–1827, 1960.
34. Weissman B: Genetic behavior of tumorigenticity in human cancer. Cancer Surv 9:475–485, 1990.
35. Knudson AG Jr.: Mutation and cancer: Statistical study of retinoblastoma. Proc Natl Acad Sci USA 68:820–823, 1971.
36. Knudson AG, Meadows AT, Nichols WW, Hil R: Chromosomal deletion and retinoblastoma. N Engl J Med 295:1120–1123, 1976.
37. Sparkes RS, Murphree AL, Lingua RW, Sparkes MC, Field LL, Funderburk SJ, Benedict WF: Gene for hereditary retinoblastoma assigned to human chromosome 13 by linkage to esterase D. Science 219:971–973, 1983.
38. Benedict WF, Murphree AL, Banerjee A, Spina CA, Sparkes MC, Sparkes RS: Patient with 13 chromosome deletion: Evidence that the retinoblastoma is recessive cancer gene. Science 219:973–975, 1983.
39. Cavenee WK, Dryia TP, Phillips RA, Benedict WF, Godbout R, Callie BL, Murphree AL, Strong LC, White RL: Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. Nature (London) 305:779–784, 1983.
40. Friend SH, Bernards R, Rojeli S, Weinberg RA, Rapaport JM, Alberts DM, Dryja TP: A human DAN segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature (London) 323:643–646, 1986.
41. Goodrich D, Lee WH: The molecular genetics of retinoblastoma. Cancer Surv 9:529–553, 1990.
42. DeCaprio JA, Ludlow JW, Figge J, Shew JY, Huang CM, Lee WH, Marsilio E, Paucha E, Livingston DM: SV40 large T tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell 54:275–283, 1988.
43. Whyte P, Buchkovich KJ, Horowitz JM, Friend SH, Raybuck M, Weinberg RA, Harlow E: Association between an oncogene and an anti-oncogene: The adenovirus E1A proteins bind to the retinoblastoma gene product. Nature 334:124–129, 1988.
44. Chellappan SP, Hiebert S, Mudryi M, Horowitz JM, Nevins JR: The E2F transcription factor is a cellular target for the RB protein. Cell 65:1053–1061, 1991.
45. Bagchi S, Weinmann R, Raychaudhuri P: The retinoblastoma protein copurifies with E2F-I, and E1A-regulated inhibitor of the transcription factor E2F. Cell 65:1063–1072, 1991.
46. Huang HJS, Yee JK, Shew JY, Chen PL, Bookstein R, Friedmann T, Lee EY-HP, Lee HW: Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. Science 242:1563–1566, 1988.
47. Abromson DH, Ellsworth RM, Kitchin FD, Tung G: Second nonocular tumors in retinoblastoma survivors. Ophthalmology 91:1351–1355, 1984.
48. Hollstein M, Sidransky D, Vogelstein B. Harris CC: p53 mutations in human cancers. Science 253:49–53, 1991.
49. Malkin D, Li FP, Strong LC, Fraumeni JF Jr., Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA, Friend SH: Germline p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science 250:1233–1238, 1990.
50. Viskochil D, Buchberg AM, Xu G, Cawthon RM, Stevens J, Wolff RK, Culver M, Carey JC, Copeland NG, Jenkins NA, White R, O'Connell PO: Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. Cell 62:187–192, 1990.
51. Wallace MR, Marchuk DA, Andersen LB, Letcher R, Odeh HM, Saulina AM, Fountain JW, Brereton A, Nicholson J, Mitchell AL, Brownstein BH, Collins FS: Type 1 neurofibromatosis gene: Identification of a large transcript disrupted in three NF1 patients. Science 249:181–186, 1992.
52. Xu G, O'Connell P, Viskochil D, Cawthon R, Robertson M, Dunn D, Stevens J,

Gesteland R, White R, Weiss R: The neurofibromatosis type 1 gene encodes a protein related to GAP. Cell 62:599–608, 1990.
53. Trahey M, McCormick F: A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. Science 238:542–545, 1987.
54. Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Dral A, Yeger H, Lewis WH, Jones C, Housman DE: Isolation and characteriztion of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor gene locus. Cell 60:633–640, 1990.
55. Fearon ER, Cho KR, Nigro JM, Kern Se, Wimons JW, Ruppert JM, Hamilton SR, Preisinger AC, Thomas G, Kinzler KW, Vogelstein B: Identification of a chromosome 18q gene that is altered in colorectal cancers. Science 247:49–56, 1990.
56. Kinzler KW, Nilbert MC, Su LK, Vogelstein B, Bryan TM, Levy DB, Smith KJ, Preisinger AC, Hedge P, McKechnie D, Finniear R, Markham A, Groffen J, Boguski MS, Altschul SF, Horii A, Ando H, Miyoshi Y, Miki Y, Nisgisho I, Nakamura Y: Identification of FAP locus genes from chromsome 5q21. Science 253:661–665, 1991.

2. Activated oncogenes and putative tumor suppressor genes involved in human breast cancers

Debasish Tripathy and Christopher C. Benz

Introduction

The clinical progression of human breast cancer almost certainly reflects accumulated molecular defects in specific genes that are important in regulating normal breast tissue growth and development. These specific defects may occur in association with gross chromosomal damage and aberrations in total cellular DNA content, once providing the only basis for their genetic detection. Within the past decade, however, new molecular techniques have enabled the detection of an increasing number of genetic abnormalities that are specific to malignant cells but not necessarily unique to breast cancers. Some of these molecular defects found in the earliest stages of human breast cancer (e.g., c-*erb*B-2 amplification) appear to have clinical significance by their ability to predict tumor relapse and patient survival, suggesting that they produce permanent cellular disturbances contributing to the proliferative or invasive nature of breast cancer. Nonetheless, a unified molecular model explaining breast carcinogenesis and its clinical progression is far from being realized. As this review demonstrates, we have only begun to identify and characterize clinically significant genetic abnormalities as they are found in primary human breast cancers. At present, it is useful to classify these molecular abnormalities into two types: gain-of-function genetic events that activate proto-oncogenes by DNA mutation, rearrangement, or amplification, and loss-of-function defects reflecting putative tumor suppressor genes that have been inactivated by DNA mutation and unmasked by deletion or allelic loss.

Karotypic abnormalities in human breast tumors

Well before the development of molecular techniques that have begun to alter our pathogenetic classification of malignancies, cytologic descriptions of aneuploid DNA content and nonrandom karyotypic abnormalities in breast tumors focused the attention of investigators on possible chromosomal defects underlying breast tumorigenesis. The accumulated database of

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breast cancer karyotypic abnormalities suffers from the lack of any large series looking at direct tumor cell preparations with modern banding techniques. Highly abnormal karyotypes are commonly seen in direct cell harvests or short-term cultures of human breast cancer cells, whereas long-term cultures and established cell lines often yield normal diploid karyotypes [1]. Karyotypic variations between cells in a given tumor or among different tumor specimens also make it difficult to separate out random from nonrandom chromosomal changes that may be systematically overrepresented and of potential biological importance. The proportion of breast cancer specimens yielding sufficient metaphase spreads suitable for banding analysis is approximately 20%. Despite these limitations, consistent reports of nonrandom abnormalities involving chromosomes 1, 3, 6, 7, and 11 have been noted using standard cytogenetic techniques.

Chromosome 1 contains the most frequently detected cytologic abnormalities in breast cancers, with up to 80% of analyzable specimens showing nonrandom abnormalities, usually consisting of translocations and rearrangements with short-arm (lp) losses and long-arm (lq) gains [2,3]. Rearrangements in chromosome 1 exhibit breakpoint clustering in two distant regions, usually involving 1p11–13 and 1q21–23 [4]. Chromosome 3 alterations have been seen in 40% of specimens, with clustering of these abnormalities around 3p11–14, similar to observations in renal cell and small cell lung cancers [4]. Chromosome 11 changes have been documented in over 60% of analyzable breast tumors with the regional involvement of 11q23–34 and 11p15 bands [5]. Nonrandom losses and rearrangements have also been noted to variable extents in other chromosomes, including 4, 6q, 7p, 8p, 9p, 13, 16q, 17p, 18, and 19 [1,2,6].

Table 1. Loss of heterozygosity (LOH) frequency in human breast cancer

Chromosome location of lost allele	Potentially involved tumor suppressor or nearest known gene	LOH frequency	Reference
1p		10–41%	12,13,29
1q	DF3/*pum*	21–41%	12,17,21,22,29
3p	*raf*-1, *erb*Aβ, *erb*A2	24–47%	12,27–29
6q		48%	12
7q	c-*met*	41%	35
11p	Ha-*ras*, β-globin, PTH	20–29%	28,29,37,38
13q	*Rb*-1	26–40%	12,28,29,43
16q		38–51%	12,29,48
17p	p53	27–58%	12,29,50,65,367
17q	*nm23*	24–64%	12,29,69
	Estradiol 17β-dehydrogenase		
	GHC		
	*erb*B-2		
	RARα		
18q	DDC	2.5–28%	12,29,74,367
22q	BCR1	27%	29
	IGLV		

Homogeneously staining regions (HSRs), perhaps representing large stretches of amplified DNA, have been noted on chromosomes 8, 19, and 20; altogether 15 of 44 (36%) tumors in one series exhibited HSRs [6,7]. Double minutes (DMs), perhaps carrying amplified DNA sequences that are not chromosomally integrated, have been seen in 48% of 50 primary breast tumors and in 40% of 46 metastatic pleural effusions [8]. In other large series, however, the presence of DMs have not been demonstrated [6,9]. Furthermore, HSRs and DMs identified to date have not been directly associated with any of the oncogenes known to be amplified in human breast cancers [7,10,11].

In brief, breast cancer karyotypic changes may localize either loss-of-function or gain-of-function genetic abnormalities. Those involving chromosomes 1, 3, 6, 11, 13, 16, 17, and 18 are most consistent with loss of genetic sequences, as determined by molecular analysis, perhaps pointing to loci of inactivated tumor suppressor genes.

Loss-of-function defects: Allelic loss unmasking an inactivated tumor suppressor gene

The use of restriction fragment length polymorphism (RFLP) analysis permits the identification of lost allelic DNA sequences (loss of heterozygosity, LOH) in a breast cancer sample when the patient's normal DNA is 'informative,' or possessing polymorphic allelic sequences at that chromosomal site. For some tumors, LOH loci correlate with karyotypic abnormalities, but in others this is not the case, presumably due to the relatively small genomic segment that can be detected as an allelic loss by RFLP analysis. The concurrent finding of a mutation in the nondeleted allele confirms the location of a putative tumor supressor gene at that genetic locus; and in some instances these LOH loci map closely to known or suspected tumor suppressor genes, as shown in Table 1. Recently a model was proposed suggesting that breast cancers accumulate losses at multiple genetic loci in a stepwise fashion, correlating with more malignant and metastatic tumor cell behavior [12].

Chromosome 1 LOH at 1p36 has been noted in breast tumors with a frequency approaching 40% by Genuardi et al., and this association appears to occur more frequently in patients with a strong family history of breast cancer, young age, and multifocal disease at diagnosis [13]. LOH at this chromosomal region has also been noted in medullary thyroid carcinomas and pheochromocytomas in patients with multiple endocrine neoplasia Type 2 (MEN2), although the MEN2-predisposing locus is independently associated with chromosome 10 LOH by linkage analysis [14–16]. LOH at 1q23–32 occurs in 20–25% of breast tumors examined [17,18]. A polymorphic locus within 1q21–23 encodes several mucinlike glycoproteins. One such gene, DF-3/*pum*, produces a 300-kDa glycoprotein and contains a

region with a variable number of tandem repeats (VNTR region) within one of its exons [19,20]. Merlo et al. and Gendler et al. used this polymorphism for the RFLP analysis of 133 breast tumors and found that 35% of tumors have lost one allele at this locus; many of these LOH events occurred as a result of internal deletions that seemed to be associated with decreased expression of the DF-3 glycoprotein [21,22]. The biological significance of DF-3 and its reduced expression due to 1q21–23 LOH remains unknown, especially in light of a recent report by Chen et al. showing that the most frequent region of LOH within 1q21–23 does not include DF-3 [23]. However, other studies are consistent with the possibility that DF-3 represents a tumor suppressor gene product. Normally, DF-3 glycoprotein is expressed on the apical borders of nonsecretory mammary cells, but staining with an anti-DF-3 monoclonal antibody reveals the loss of this membrane-staining polarity in a majority of breast cancers [24]. Increased DF-3 expression tends to correlate with well-differentiated tumors and estrogen receptor (ER) positivity [25]; furthermore, one retrospective study involving 190 patients with stage II breast cancer demonstrated superior disease-free and overall survival in patients whose tumors showed high DF-3 immunoreactivity [26].

LOH on chromosome 3 within 3p21–25 is also seen in about 30% of informative primary breast tumors [12,27–29]. This genetic abnormality correlates with c-*myc* overexpression, chromosome 11 deletions, high histopathologic tumor grade, ER negativity [30], and DNA aneuploidy [29]. The shortest overlapping region of deletion within 3p23–25 seems to include both c-*erb*Aβ, a thyroid hormone receptor encoding gene, and c-*erb*A2, a related gene whose function is unknown [27,28]. This same pattern of LOH is seen in lung cancer, familial and sporadic renal cancer, and von Hippel-Lindau disease, with its inherited susceptibility to multiple cancers [31–33]. Allelic loss at this locus is intriguing, since the thyroid hormone receptor is important for both tissue development and differentiation, and a loss of receptor function by either gene mutation or deletion could lead to constitutive repression of receptor-mediated differentiation [34], a potentially tumorigenic event. The serine-threonine kinase protooncogene, c-*raf*1, also maps within this chromosome 3 region; however, LOH at the c-*raf*1 locus appears to occur at a much lower frequency [27].

Allelic loss at several loci on chromosome 6q has been noted in 48% of breast cancers in one series, and this accumulation of LOH events appears to be inversely correlated with the number of axillary lymph nodes involved with tumor [29]. Bieche et al. noted LOH at chromosome 7q31 in 41% of 121 informative primary breast cancers using a probe for the c-*met* proto-oncogene, and this LOH correlated with shortened metastasis-free and overall survival [35]. The c-*met* gene encodes a tyrosine receptor kinase recently shown to be the hepatocyte growth factor receptor [36]. Although its significance in breast cancer remains unclear, c-*met* or a nearby gene may be a candidate tumor suppressor gene.

For chromosome 11p, LOH is evident in about 25% of informative breast tumors, and most studies have focused on the distal end of this chromosome at 11p15, which includes the polymorphic c-Ha-*ras* locus. At least two groups have documented a correlation between allelic loss at this locus and increased primary tumor size, ER negativity, and high histologic grade [37,38]. There appears to be no difference in the distribution of rare and common c-Ha-*ras* alleles in breast tumor specimens as compared to normal tissue controls [37,39–41], and no point mutations in the known *ras*-activating codons of the remaining allele [42]. Gain-of-function *ras*-activating mutations will be discussed later; however, in breast tumors expression of the c-Ha-*ras* transcript and $p21^{ras}$ protein product occurs at higher than normal tissue levels, whether or not there is LOH at this genetic locus [42]. More detailed RFLP analysis has revealed that the most frequently deleted 11p15 sequence occurs centromeric to the c-Ha-*ras* locus, probably explaining the lack of any direct association between 11p15 LOH and c-Ha-*ras* activation in breast tumors and pointing to the putative existence of a tumor suppressor gene lying between the β-globin and parathyroid hormone (PTH) genes within 11p15 [38].

Chromosome 13 LOH was initially shown to occur at several different loci [43]. Subsequent to the identification of the retinoblastoma tumor suppressor gene (Rb-1) at chromosome 13q14.1, several groups have observed abnormalities in the Rb-1 gene that include homozygous internal deletions and duplications, resulting in absent or aberrant Rb-1 transcripts and loss of Rb-1 protein expression [44,45]. In one large series of primary breast tumors, deletion or rearrangement of the *Rb*-1 gene was seen in 15/77 (19%) of tumors, with no *Rb*-1 abnormalities detected in lymphocytes or benign breast lesions from the same patients [46]. Those tumor cases with altered *Rb*-1 genes also showed negative immunohistochemical staining for the *Rb*-1 protein in at least a proportion of the tumor cells. Some tumors with no structural changes in the *Rb*-1 gene demonstrated absent *Rb*-1 protein expression, suggesting that breast tumors may also develop abnormalities in the transcriptional regulation of an otherwise intact *Rb*-1 coding sequence or that alternative mechanisms may lead to *Rb*-1 protein sequestration and degradation. At present, the relative importance of *Rb*-1 defects in breast cancers remains uncertain, yet patients carrying the *Rb*-1 defect in association with hereditary retinoblastoma seem to be at increased risk for the development of breast cancer [47], supporting the possibility that inactivation of this tumor suppressor gene represents a critical event in the multistep progression to invasive breast cancer.

Allelic loss at 16q has been found to occur in over 40–50% of primary breast tumors [12,29], and this defect appears to associate with breast tumors having nodal metastases [48]. With over 90% of this chromosome now mapped by the Human Genome Project, increased attention will be given to the search for a putative 16q breast tumor suppressor gene.

The most frequent RFLP finding in human breast tumors studied to date,

however, is LOH at multiple loci on chromosome 17p. 17p deletions are also common in small cell lung, colon, and ovarian cancers as well as osteosarcomas [49 and references therein]. Coles et al. probed multiple loci from 17p13.1 to 17p13.3 in 168 breast tumors and noted LOH in nearly 60% of informative cases. The most commonly involved locus was found to be 17p13.3, and the tumor suppressor gene p53 (located on 17p13.1) exhibited LOH in only 27% of cases, introducing the possibility of another breast tumor suppressor gene located telomeric to p53. Others have noted breast cancer associated LOH extending from 17p12 to 17p13.3 [12,29,50]. Sato et al. noted that 17p13.3 LOH, when combined with 13q LOH in a given breast tumor, was associated with a more malignant histology [48]; additional reports note a correlation of 17p allelic loss with DNA aneuploidy [29,51].

Recent attention has been given to p53 gene abnormalities and protein overexpression in breast cancers as well as other malignancies. Circulating anti-p53 antibodies detected in patients with breast cancer first suggested an important clinical role for the p53 phosphoprotein [52]. Subsequently, overexpression of p53 protein has been noted in 15–27% of breast cancer specimens [53–60]. p53 overexpression is seen both in in situ and invasive breast cancer, and the same staining pattern observed in primary compared to metastatic lesions in a given patient, suggesting that this change is an early and sustained event in breast tumorigenesis [61]. There is a close correlation between p53 overexpression, loss of heterozygosity at or near the p53 locus, and mutations in the remaining p53 allele [59,62–64], although overexpression without p53 allelic loss and a single mutated p53 allele can also lead to overexpression [59]. p53 mRNA levels in tumors overexpressing p53 are not elevated, in keeping the fact that mutated p53 protein has a longer half-life, which explains its overexpression [52].

LOH at 17p13.3, which is some 20 megabases distal to the p53 gene, correlates with p53 overexpression in some series [65] but not in others [54,59]; this finding is consistent with the possibility that other genetic changes at 17p can also lead to p53 overexpression and that this may occur independent of p53 mutations or LOH. The independent clinical significance of p53 overexpression was recently shown by Thor et al., who found overexpression in primary breast tumors was associated with overall and disease-free patient survival as well as tumor ER negativity and poor histologic grade [54]. These observations are supported by other correlative studies [55–58]. In breast tumors as well as other tumor types, p53 mutations are generally single base-pair missense substitutions that fall within five evolutionarily conserved regions spanning about 600 base pairs (codons 110–327) and encompassing p53 exons 5–8 [49,62–64, see Chapter 17]. One series examining 59 breast cancer specimens found 10 (17%) mutations in exons 5–9 of the p53 gene [66]. G:C amd A:T mutations tend to predominate for most human tumor types, including breast cancer, but, in contrast to colon cancer, only a minority of these occur in CpG islands [49]. The rare Li-Fraumeni familial autosomal dominant syndrome with early

predisposition to breast cancer, soft tissue sarcomas, brain tumors, osteosarcoma, and other malignancies is now known to involve allelic germline mutations in the p53 gene [67,68]. These germline mutations vary between families, but they are generally single base-pair missense substitutions, like those seen in the sporadic tumors, and in the one case in which tumor tissue was available for analysis, the remaining wild-type allele had been lost [67].

Specific attention has also been given to allelic losses at two chromosome 17q regions. The 17q11 locus of the *nm23* gene, whose expression is reduced in metastatic murine and human tumors, including breast cancer, shows LOH in over 60% of breast tumors as reported by Leone et al. [69]. This putative tumor suppressor gene is discussed in greater detail in Chapter 18. Investigators have also noted LOH at several more distal 17q loci with a frequency ranging from 24% to 35%, the higher frequency correlating with younger age patients [12,29,70,71]. In particular, a familial form of breast cancer occurring in young women is being linked to a potential breast cancer suppressor gene at 17q21–22, which is currently being investigated by at least three different groups worldwide (M.C. King, Berkeley, CA; G.M. Lenoir, Cedex, France; B.J. Ponder, Cambridge, UK). Known genes near this locus include c-*erb*B-2, homeobox 2, *nm23*-H1, and those encoding the retinoic acid receptor (RAR)-α (discussed later) and the progestin-inducible catabolic enzyme, estradiol-17β-dehydrogenase [72]. Loss of function of this latter enzyme would be expected to increase endogenous estradiol levels, a physiological condition associated with increased risk for breast cancer.

The DCC tumor suppressor gene, found to be inactivated in colon cancers and located on chromosome 18 within 18q21.3–23, encodes an adhesion glycoprotein that may have a role in breast cancers [73]. In human breast tumors, LOH in this 18q region has been observed in 24–38% of informative samples [29,74]. Using a probe mapping to the DCC gene itself, however, Sato et al. found only 1 of 40 breast tumors with DCC allelic loss, perhaps implicating the existence of a different 18q breast tumor suppressor gene [12].

In all, between 20% and 60% of primary spontaneous human breast cancers have allelic loss or deletion occurring on chromosomes 1, 3, 6, 11, 13, 16, 17, 18, and 22, with the exact chromosomal loci involved yet to be defined. Previous knowledge about the Rb-1 and p53 genes at 13q14 and 17p13, respectively, makes it likely that new tumor suppressor genes will be found at many if not all of these breast-cancer-involved chromosomal loci.

Gain-of-function events: DNA mutation, rearrangement, or amplification producing a dominant-acting oncogene

Genetic alterations in breast cancer leading to the activation of proto-oncogenes were initially observed in both virally and chemically induced murine mammary tumors. The mouse mammary tumor virus (MMTV), with

its *cis*-acting transcriptional activating long terminal repeat (LTR) elements, can integrate its proviral DNA into specific host genomic sites, thereby transcriptionally activating silent genes that could predispose to cellular proliferation and the development of murine mammary cancers [75]. To date, seven of these specific integration sites, or *int* genes, have been described (initially designated *int*-1, 2, 3, 4, 5, 41, and *hst*-1) [76]. The first of these genes to be discovered, *int*-1 (now designated *wnt*-1 [77]) was found to encode an evolutionarily conserved protein homologous to the *Drosophila* morphogen, *wingless* [78]; in mice, *wnt*-1 is normally expressed in a very restricted manner in embryonic neural tissue and in adult testes [79]. Transfection of activated (MMTV-LTR-promoted) *wnt*-1 into immortalized cells results in malignant transformation as determined by soft agar colony formation and tumorigenic growth in nude mice [80]. *Wnt*-1 bearing transgenic mice develop mammary gland hyperplasia and focal mammary tumors that are hormonally independent [81]. The second member of this family of genes, *int*-2, was later found to encode a member of the fibroblast growth factor (FGF) family, and this protein appears to be normally expressed in a complex pattern during murine embryogensis, suggesting that it too normally functions as a morphogen [82–85]. Upon transfection, *int*-2 also induces malignant transformation in mammalian cells [86]. Transgenic mice bearing *int*-2 constructs develop hyperplastic mammary glands following pregnancy, with tumors forming at a low frequency only after a long latency period [87]. Endogenous activation of *int*-2 by insertion of MMTV sequences produces murine mammary tumors that are initially hormone responsive but later become hormone independent [88,89]; this behavior parallels that seen in the progression of estrogen-responsive human breast cancers. To date, *int*-3, 4, 5, and 41 have not been well characterized [90,91]. The *hst*-1 gene (*hst*F1, *FGF*-K) was first described as a transforming gene found in human gastric cancers [92], Kaposi's sarcoma [93], and subsequently found to be activated in MMTV-induced murine mammary tumors [94]. It also shares structural homology with FGFs and *int*-2 [95]. Although the murine *int* genes are not syntonic (except *int*-2 and *hst*-1), they are frequently activated together, either sequentially or in various combinations, suggesting a multistep progression in MMTV-induced mammary neoplasia [82,94,96,97].

Genes amplified at 11q13

In human breast cancers, the 11q13-colocalized *int*-2 and *hst*-1 genes are either singly amplified or frequently coamplified, as seen in a variety of other solid tumors, including esophageal [98], head and neck [99], bladder [100], and hepatocellular cancers [101], and in melanoma [102]. Amplification of *int*-2 is observed in 9–23% of primary breast tumors [103–109], and this is associated with an 11q13 amplicon that commonly includes *hst*-1, which is 35 kb distant from *int*-2 [110,111]. Amplification of *int*-2 seems to

correlate with increased tendency for breast cancer metastases and locally recurrent disease [106,112], as well as decreased patient survival [99,108]. In some studies, *int*-2 amplification has been associated with more differentiated tumors, including those that are ER and PR positive [100,109]; in contrast, there has been no correlation with tumor stage or patient characteristics, such as age and menopausal status [100].

In an RFLP analysis of 96 breast cancer specimens, one particular *int*-2 allele correlated with lymph node involvement but not with any other clinical parameter [113]. In contrast to the murine model, transcriptional activation by amplified *int*-2 and *hst*-1 genes has been difficult to demonstrate. Liscia et al. detected low transcript levels of *int*-2 but not *hst*-1 by in situ hybridization and Northern blot analyses of breast tumor specimens coamplified for these two genes [114]. The breast cancer *int*-2 transcripts on Northern blots were not the size expected based on the *int*-2 physical map or as formerly observed in teratocarcinoma cells [104].

Theillet et al. detected low levels of both *int*-2 and *hst*-1 amplification in breast tumors, but only *hst*-1 expression correlated with gene amplification [105]. In contrast, using the more sensitive RNA protection assay, Fantl et al. were unable to detect *int*-2 or *hst*-1 transcripts in either gene-amplified or unamplified tumors [104]. Therefore, the biological role of amplified *int*-2 and/or *hst*-1 genes, which may or may not be transcriptionally activated in human breast cancers, remains unclear.

Given this uncertainty, more recent attention has focused on other genes that are frequently coamplified with *int*-2 and *hst*-1 within the 11q13 amplicon. The *bcl*-1 locus at 11q13 was initially described as a common breakpoint in B-cell chronic lymphocytic leukemia bearing the t(11;14) chromosomal translocation [115,116]. It is located at least 1000 kb centromeric to the *int*-2 and *hst*-1 genes [110]. Ali et al. showed coamplification of *int*-2, *hst*-1, and *bcl*-1 in 17 out of 18 breast cancer specimens, with one specimen showing only *int*-2 amplification [117]. Theillet et al. found 17% of 297 samples coamplified for *bcl*-1, *int*-2, and *hst*-1; however, *int*-2 and *hst*-1 were never amplified independently of *bcl*-1, and in six cases *bcl*-1 alone was amplified [118]. The *sea* oncogene, also found at 11q13, and the *ets*-1 gene, found at 11q24, were each amplified in only 3 of the 297 cases.

These results indicated that the most frequent loci of 11q13 amplification in human breast cancers include *int*-2, *hst*-1, and *bcl*-1, but not *sea*; furthermore, the gene of real interest in the 11q13 amplicon may lie closer to *bcl*-1 than to *int*-2 or *hst*-1. A gene linked to the *bcl*-1 marker at a distance of less than 250 kb (D11S287 or *PRAD*-1) was initially noted to be clonally rearranged in a subset of parathyroid adenomas [119] and subsequently was found to be coamplified with *int*-2 and *hst*-1 in 18% of 202 primary breast cancers, including one case in which the amplification unit did not extend to *bcl*-1 [120]. Furthermore, a 4.5 kb mRNA transcript was readily detectable in the tumors exhibiting *PRAD*-1 amplification, but not in other tumors or normal mammary tissue. Using chromosome walking techniques, Motokura

et al. and Withers et al. have cloned and sequenced this gene and have found it to encode a novel D1 (G1-type) cyclin [121,122]. This breast-cancer-amplified protein exhibits cyclin function in that it can bind a p34/cdc2-like protein (which is distinct from cdc2) [123] and activates histone H1 kinase activity [121].

Three other groups have identified the same gene through its ability to complement bacterial mutants for all three of the known G1-type cyclins [123–125]. Faust et al. noted overexpression of this gene in 5 out of 7 breast cancer cell lines using Northern blot analysis, only moderately correlating with gene amplification, but not correlating with the rate of cell growth in culture [126]. Therefore, it is likely that the cyclin D1 gene (*PRAD*-1) plays a pivotal growth-promoting role in the nearly 20% of human breast cancers having amplification at 11q13, although overexpression in the absence of amplification suggests transcriptional control as well. The potential link between cyclins and various human malignancies, including breast tumors, remains intriguing, and the possibility that D1 cyclin can function as a transforming oncoprotein must be investigated further.

The gene encoding an isozyme of glutathione S-transferase, GSTπ, is also located at 11q13, distal to *bcl*-1 [127], and increased expression of this detoxifying enzyme is known to account for some forms of tumor resistance to antineoplastic agents, such as melphalan and doxorubicin [128–130]. Doxorubicin resistant breast cancer cell lines are known that preferentially express GSTπ, as has been observed in some surgically resected human lung, head and neck, colon, and breast cancers [128,131–133], and preferential expression has been noted in sarcomas with biologically aggressive features [134]. Of note, some GSTπ-overexpressing and doxorubicin-resistant breast cancer cells do not exhibit GSTπ gene amplification [135]. However, Saint-Ruf et al. found GSTπ amplification in 6 out of 6 breast cancer samples having either *int*-2 or *hst*-1 amplification, but not in 11 samples without 11q13 amplification [101]; unfortunately, tumor cell expression of GSTπ was not assessed in this study. In contrast, Theillet et al. were unable to detect GSTπ amplification in a subset of 22 breast cancers having *int*-2, *hst*-1, and *bcl*-1 amplification [118]. Thus, it remains to be established whether or not breast tumors with 11q13 amplicons also demonstrate GSTπ amplification, overexpression, or resistance to chemotherapeutic agents such as doxorubicin.

Amplification of c-myc

The transcriptional activation of the c-*myc* proto-oncogene by gene rearrangement or amplification has long been implicated in tumor initiation and progression, and c-*myc* overexpression has been observed in various human malignancies, including lymphoma [136,137], leukemia [138,139], lung [140], colon [141], and breast [142] cancers. However, the possible role of c-*myc* overexpression in breast tumor progression is confounded by its known

secondary induction in normal and malignant cells exposed to mitogens, growth factors, and various other stimuli [143–146]. Transgenic mice bearing an MMTV long terminal repeat (LTR) fused to c-*myc* coding sequences develop hormone-dependent solitary breast cancers, testicular tumors, as well as B-cell, T-cell, and mast cell tumors [147,148]. When c-*myc* is fused to a lactogenic (whey acidic protein, WAP) promoter in a transgenic mouse model, premalignant changes precede the development of multifocal mammary adenocarcinomas, forming only after pregnancy [149]. Both of these transgenic c-*myc* models implicate the need for one or more additional tumorigenic steps besides c-*myc* activation; these additional steps may be facilitated by the hormonal dependency of the WAP and MMTV promoters.

In an estrogen-dependent human breast cancer cell line, MCF-7, the transfection of a murine leukemia virus LTR-c-*myc* exon 2–3 construct resulted in slower in vitro cell growth and no change in the cell line's in vivo tumorigenicity or hormone dependency, calling into question the ability of overexpressed c-*myc* sequences to enhance breast cancer progression [150]. Estrogen-responsive MCF-7 and T47-D cells exhibit rapid induction of c-*myc* mRNA upon treatment with estrogen; and the antiestrogen, tamoxifen, causes a decline in c-*myc* mRNA [151–153]. Furthermore, antisense oligodeoxynucleotides to c-*myc* have been shown to selectively inhibit MCF-7 *myc* protein expression in response to estrogen, which is accompanied by an inhibition in estrogen-stimulated cell growth; however, these same antisense oligomers inhibit the estrogen-independent growth of MDA-MB-231 cells [154]. All of the above studies support the fact that enhanced expression of c-*myc* may be necessary for breast cancer growth but is not necessarily sufficient for breast cancer induction or progression. Constitutive overexpression resulting from amplification of a proto-oncogene, such as c-*myc*, must also be distinguished from the enhanced growth-factor-induced proto-oncogene expression that can be seen during short-term culture growth of normal breast epithelial cells [155].

Amplification of c-*myc* was initially observed in the human breast cancer cell lines SKBR-3 [156] and SW6B-5 [157]; in the latter case it was associated with the cytogenetic findings of DMs and HSRs. In an early review of proto-oncogene expression in primary human tumors, Slamon et al. noted that c-*myc* transcript levels were almost uniformly elevated in a variety of solid and hematologic tumors, including four cases of breast cancer, although the potentially confounding influence of admixed stromal and inflammatory cells was not assessed in this study [142]. Other investigators have detected c-*myc* amplification in primary breast tumors with a frequency ranging from 4% to 41%, with c-*myc* gene rearrangements occuring in fewer than 16% of cases [109,158–167].

The clinical implications of c-*myc* amplification have been studied by restrospective analysis of archival samples, and these studies have produced inconclusive results. Reports have suggested that amplification correlates

with patient age <50 [158], poor survival [162], tumor nodal metastases [112,164], progesterone receptor (PR) negativity [109], high cathepsin-D expression [167], inflammatory histology [165], and poor tumor grade [166]. However, these relationships have not been observed consistently, and other reports indicate that they may only be apparent when c-*myc* amplification occurs in association with other amplified proto-oncogenes, such as c-Ha-*ras* or c-*erb*B-2 [161]. If, in fact, there is no clinical significance to the finding of c-*myc* amplification, it is difficult to understand what the selective cellular pressures are that account for the increased c-*myc* gene copies found in up to 40% of human breast cancers.

Overexpression of c-*myc* mRNA and protein generally result from gene amplification; however, they can also occur in the absence of either gene amplification or rearrangement, as suggested earlier [158,162]. Elevated c-*myc* mRNA transcript levels have been observed in 45% of breast tumors in one study using in situ RNA:RNA hybridization, and this overexpression was correlated with nodal involvement by tumor [168]. Using a similar technique, LeRoy et al. demonstrated lower c-*myc* mRNA expression in ER-positive tumors treated preoperatively with tamoxifen, as compared to a similar group of untreated tumors, again suggesting that c-*myc* expression correlates with tumor growth in an estrogen-dependent fashion [169].

The 62-kDa *myc* oncoprotein can be detected by immunohistochemical staining and in one study was observed in 70% of 100 tumors, although this expression was not found to correlate with any clinical parameters [170]. Locker et al. measured c-*myc* protein levels by flow cytometry and noted a clinical association only with tumor differentiation [171]. High levels of *myc* staining in surgical breast cancer specimens is usually apparent only in malignant cells and not in the surrounding normal breast or stromal cells [172]. In one report examining benign fibrocystic disease, however, detectable *myc* staining was not seen in the normal epithelial cells, but strong staining was present in the metaplastic cells within benign epitheliosis and papillomatous lesions [173]. In contrast, Spandidos et al. observed high *myc* staining in normal breast tissue adjacent to tumor and speculated that its induction in the normal cells might be due to paracrine factors secreted by the tumor cells [174].

Activated ras *oncogenes*

The c-Ha-*ras*, c-K-*ras*, and c-N-*ras* family of protooncogenes encode 21-kDa guanine nucleotide-binding proteins ($p21^{ras}$) that are able to catalyze the hydrolysis of GTP and thereby stimulate intracellular signal transduction [reviewed in 175]. Activating point mutations in *ras* codons 12, 13, and 61 prevent the interaction of $p21^{ras}$ with GTPase-activating protein (GAP), maintaining $p21^{ras}$ in an activated and GTP-bound form. Such *ras* mutations have been consistently noted in colon [176,177], pancreas [178], and lung cancers [179], but not in human breast cancers [42,180]. In contrast, these

mutations are frequently found in carcinogen-induced rodent mammary carcinomas, and activated *ras* oncogenes readily produce mammary cancers in transgenic mouse models.

N-nitroso-N-methylurea (NMU) will consistently induce estrogen-dependent mammary cancers in female rats after a 6 to 12-month delay. Over 80% of these mammary tumors contain a GGA to GAA mutation in codon 12 of the c-Ha-*ras* gene, in keeping with the ability of NMU to methylate the N^7 and O^6 positions, thus causing G to A mutations [181]. Similarly, 7,12-dimethylbenz(a)-anthracene (DMBA)-induced rat mammary tumors exhibit c-Ha-*ras* mutations in codon 61 in 25% of cases [182]. In contrast to this sex-dependent induction of malignancy, both male and female MMTV LTR/v-Ha-*ras* transgenic mice develop focal mammary tumors in a stochastic fashion, with the less frequent development of salivary gland tumors, lymphoma, and lacrimal gland hyperplasia [148]. Transgenic progeny of MMTV LTR/c-*myc* and MMTV LTR/v-Ha-*ras* crosses yield similar tumors that develop much more rapidly [148], supporting the cooperative potential of these two transforming constructs. A WAP promoter/activated c-Ha-*ras* construct produces transgenic females that develop solitary breast and salivary gland tumors at a low frequency and after a long latency [183].

With each of these different *ras* transgene constructs in which the promoter is altered to produce different mouse transgenic strains, there is a positive correlation between the frequency of tumor development and the level of *ras* expression [184]. The stochastic manner in which these focal tumors arise suggests that further genetic changes are necessary, in addition to *ras* activation, for the full development of mammary cancer. In one interesting in vitro transfection study, the insertion of v-Ha-*ras* into estrogen-dependent MCF-7 cells produced estrogen-independent MCF-7 tumor growth in nude mice [185], providing a posible explanation for the pattern of in vivo tumor progression in patients whose hormone-dependent breast cancers later become resistant to endocrine therapy. A similar study suggested that c-Ha-*ras* activation can also induce resistance to cisplatin chemotherapy, possibly by increased metallothionine content or impairment of intracellular drug accumulation [186].

In spontaneous human breast cancers, however, the role of the *ras* family of protooncogenes remains uncertain. Neither amplification nor rearrangement of c-Ha-*ras* has been detected in a database of over 500 accumulated cases of primary breast cancer [42,159,161,162,165,187]. The c-Ha-*ras* gene is highly polymorphic due to its 3′ VNTR, and the distribution of its four major RFLP-determined alleles and one of its minor variants does not seem to differ in patient populations with or without breast cancer [37,39–41]. However, one study found that a rare c-Ha-*ras* allele was overrepresented in breast cancer patients as compared to controls [188]. The significance of this epidemiologic finding is unknown, as is its possible association with the c-Ha-*ras* LOH data presented earlier [37,42,165]. Unlike the carcinogen-

induced rodent mammary tumor models, activating mutations in K-, Ha-, and N-*ras* at codons 12, 13, and 61 are rarely found in human breast cancers of any stage [42,180]. Despite the apparent genetic integrity of the *ras* family of protooncogenes, overexpression of c-Ha-*ras* (but not K-*ras* or N-*ras*) can be seen in up to 70% of spontaneous human breast cancers [42,189,190]. Rarely, K-*ras* or N-*ras* overexpression is observed [142,191]. An association between elevated c-Ha-*ras* mRNA levels and advanced histologic tumor grade has been reported [192].

Archival breast tumors have been studied for $p21^{ras}$ protein expression by immunocytochemical or Western blotting techniques using monoclonal antibodies that do not distinguish between the different *ras* protein products. Overexpression of $p21^{ras}$ has been observed in 21–83% of cases [170,187,191,193–202], with the wide variation in this observed incidence probably due to different antibodies, techniques, and threshholds for positivity used by the different investigators. Most studies have shown that $p21^{ras}$ is overexpressed in both malignant and benign breast neoplasms but not in normal breast tissue.

Candlish et al., however, found no significant difference in $p21^{ras}$ staining between breast cancers, benign breast tumors, or the normal breast tissue adjacent to tumors using the same antibody [203]. Other studies have noted that the incidence of $p21^{ras}$ expression is highest in malignant breast tumors (63–83%), lower in breast fibroadenomas and cystic disease (23–42%), and lowest ($\leq$10%) in normal breast tissue [194,195,204]. As well, increased $p21^{ras}$ staining has been noted in the high-risk subgroup of benign breast disease that is composed of hyperplasia with atypia; in fact, after a 15-year follow-up, the higher $p21^{ras}$-expressing lesions were found in those patients who subsequently developed breast cancer [195].

More intense staining observed in the invasive component of breast cancers, as compared to either intraductal lesions or distant metastases, suggested to one group of investigators that *ras* expression is an early determinant in the development of invasive breast cancer [205]. Other investigators have suggested that increased $p21^{ras}$ correlates with reduced disease-free and overall patient survival [196,198,199], higher tumor stage [196], estrogen and epidermal growth factor receptor overexpression [200], c-*erb*B-2 oncoprotein overexpression [199], and younger patient age at diagnosis [170]. On the other hand, some investigators have found no clinical association with $p21^{ras}$ expression in breast tumors [206]. It seems plausible, therefore, that $p21^{ras}$ overexpression occurs secondary to other growth-stimulatory mechanisms that may become dysregulated in the earliest stages of invasive disease, or perhaps even in the most proliferative forms of preinvasive breast lesions.

*Activation of c-*mos, *c-*fos, *c-*fes, *c-*myb, *and others*

There are other potentially activated proto-oncogenes in human breast tumors that have received much less attention. The c-*mos* proto-oncogene is

located on chromosome 8p1; its product is a 39-kDa serine/threonine kinase that may function as a maturation and meiotic arrest factor in oocytes [207,208]. Although HSRs and abnormal banding patterns at 8p1 have been reported in 13% of breast tumors [9], amplification or overexpression of c-*mos* has not been reported. Of unknown significance is the rare 5-kb *Eco*RI c-*mos* allele found in both tumor and normal DNA of 8% of breast cancer patients [209,210], an incidence over 15-fold higher than that observed in unaffected populations [211].

Overexpression of the c-*fos* proto-oncogene has been seen in almost all tumor types, including breast cancer [142,190]. Its transcriptional induction, however, is a common endpoint for intracellular signaling initiated by a wide variety of cell stimuli, including stress; exposure to serum, mitogens, and growth factors; as well as differentiation and tumor-promoting agents [reviewed in 212]. Therefore, a primary role for c-*fos* involvement in breast tumorigenesis remains dubious.

Overexpression of the receptor tyrosine kinase-encoding c-*fes* and c-*fms* proto-oncogenes have been noted more selectively in some tumors, including breast, lung, and renal cancers [142]. One instance of c-*fes* amplification has also been reported in breast cancer [11].

The nuclear protooncogene, c-*myb*, appears to be overexpressed primarily in hematologic malignancies; however, Sorokine et al. noted increased anti-*myb* antibody in the sera of 43% of breast cancer patients, although this finding did not appear to correlate with tumor cell expression of c-*myb* [213]. Guerin et al. found expression of a 3.5-kb c-*myb* transcript in 64% of 169 primary breast cancers and noticed a correlation with more favorable clinical features, including low histopathologic tumor grade, ER and PR positivity, absence of inflammatory carcinoma, and lack of c-*erb*B-2 overexpression [214].

Adnane et al. noted amplification of the FGF receptor genes, *BEK* and *FLG*, in 11.5% and 12.7% of 387 breast tumors, respectively. In particular, *FLG* amplification correlated with nodal involvement and also *int*-2/*hst*-1/*bcl*-1 amplification, while *BEK* amplification correlated with c-*myc* amplification [215]. The significance of FGF receptor amplification and/or overexpression must be studied further.

Amplification of c-erb*B-2 (HER2*/neu*)*

The c-*erb*B-2 (HER2/*neu*) oncogene was first identified as the human homologue of the transforming oncogene, *neu*, in nitrosourea-induced rat brain tumors [216]. The c-*erb*B-2 proto-oncogene is located on chromosome 17q21 and encodes an approximately 185-kDa glycoprotein ($p185^{erbB\text{-}2}$) that bears extensive homology to the 175-kDa epidermal growth factor receptor (EGFR). Both are subclass I transmembrane receptor tyrosine kinases related to the truncated transforming oncogene, v-*erb*B, from the avian erythroblastosis virus [217–223]. Since the ligand for c-*erb*B-2 has not yet been well characterized, it is unclear how much of the receptor's cell

signaling is constitutive and how much is ligand dependent in receptor-overexpressing breast tumors.

In c-*erb*B-2 amplified cell lines, receptor autophosphorylation and internalization, as well as phosphatidylinositol turnover and induction of early response genes such as c-*fos*, all occur within minutes of extracellular receptor binding by agonists such as the murine monoclonal antibody, muMAb4D5 [224,225]. In the rat model, the activated *neu* gene has been shown to contain a mutation that is necessary for it to confer malignant transformation; this mutation substitutes valine for glutamic acid in the transmembrane domain [226,227], predisposing the receptor to ligand-independent dimerization and activation [228].

Transgenic mice bearing the activated *neu* gene under the control of a MMTV-LTR promoter develop synchronous, polyclonal adenocarcinomas that replace all normal mammary tissue in both male and female progeny [229]. This result is unlike that produced by the earlier discussed transgenes containing c-*myc*, v-Ha-*ras*, c-Ha-*ras*, *wnt*-1, or *int*-2, each of which produce mammary tumors in a stochastic fashion dependent on a prolonged hormonal stimulus such as pregnancy [81,147–149,183].

Curiously, a similar *neu* transgenic strain, differing only by much closer proximity of the oncogene to its MMTV-LTR promoter, produced stochastic and asynchronous growth of less aggressive tumors adjacent to normal mammary epithelium [230], suggesting that in this particular model additional genetic events were necessary for breast tumorigenesis. Like the others, these transgenic models also exhibited hyperplasia of the epididymis, seminal vesicles, and salivary glands, resulting from the tissue-specific expression of the MMTV-promoter-driven constructs.

In humans, the amplification and overexpression of c-*erb*B-2 was first noted in gastric and breast cancer cell lines as well as primary salivary gland adenocarcinomas [231–233]. Kraus et al. studied a variety of breast cancer cell lines and showed that overexpression of c-*erb*B-2 transcripts does not absolutely depend on gene amplification [234]; upstream regulatory elements have since been identified in the c-*erb*B-2 promoter, suggesting that *trans*-activating factors may be involved in the development of some c-*erb*B-2 overexpressing breast tumors [235]. The cytogenetics of c-*erb*B-2 gene amplification have not yet been fully characterized. In one cell line with c-*erb*B-2 amplification and cytogenetic evidence for DMs, in situ hybridization confirmed the chromosomal localization of the increased gene copies [10]. More recently, fluorescence in situ hybridization (FISH) indicates that c-*erb*B-2 amplified breast cancers typically exhibit intercellular heterogeneity in both the degree and pattern of chromosomal integration by the multiple gene copies; this heterogeneity is even seen within the same tumor specimen [236].

Unlike the carcinogen-induced c-*neu* from the rat brain tumor model, c-*erb*B-2 transmembrane domain mutations have not been detected in human breast, ovarian, or gastric cancers, including those with c-*erb*B-2 amplifi-

cation [237,238]. The normal human c-*erb*B-2 gene can confer a tumorigenic phenotype upon transfection and overexpression in immortalized fibroblasts [221,239]. Such c-*erb*B-2 transfectants form colonies in soft agar and develop tumors in nude mice; of interest, these overexpressing cells become resistant to the cytotoxic effects of macrophages and tumor necrosis factor-α [240]. In contrast, amino (N)-terminus truncation or point mutations introduced into the transmembrane domain of c-*erb*B-2 leads to cell transformation without the need for receptor overexpression [221,241], since the altered receptor protein possesses a higher intrinsic tyrosine kinase activity than does the normal $p185^{erbB\text{-}2}$. Furthermore, $p185^{erbB\text{-}2}$ in overexpressing primary human breast tumors is phosphorylated on tyrosine, suggesting that it is active in cell signaling [242]. Transgenic mice bearing overexpressed but normal c-*erb*B-2 develop a range of malignancies, including mammary tumors and B-cell lymphomas [243]. Transfection of wild-type, N-terminally truncated or transmembrane mutated c-*erb*B-2 into immortalized human mammary epithelial cells results in in vivo tumorigenicity that is more marked for the altered c-*erb*B-2 constructs [244]. Transfection of wild-type c-*erb*B-2 into estrogen-dependent MCF-7 cells has also resulted in low-level resistance to cisplatin and complete loss of in vivo growth inhibition by the antiestrogen tamoxifen, occurring independent of tumor ER content [245]. This particular transfection model may be analogous to the pattern of acquired tamoxifen resistance that ultimately develops in human tumors that are initially responsive to tamoxifen. From all of these basic studies, it can be surmised that amplification and overexpression of $p185^{erbB\text{-}2}$ is a necessary but not entirely sufficient genetic event, contributing to human breast tumorigenesis.

The relationship of c-*erb*B-2 amplification and overexpression to breast cancer prognosis and clinical behavior has also been widely studied. Slamon et al. initially reported 2- to 20-fold levels of gene amplification in 189 primary breast cancers, and a significant correlation between gene amplification and decreased overall survival in node-positive patients [246]. Both amplification and overexpression of c-*erb*B-2 have since been shown to correlate with reduced survival in patients with other adenocarcinomas, particularly ovarian cancer [247,248]. Overexpression of c-*erb*B-2 in the absence of gene amplification has been associated with worse prognosis for patients with lung adenocarcinomas [249]. Since the first clinical reports on c-*erb*B-2, numerous retrospective studies have been performed using archival tumor tissues and patient follow-up data in attempts to determine the actual incidence and clinical significance of c-*erb*B-2 amplification and overexpression in human breast cancers (Table 2). Pooled data from over 5000 patients shows a 20% incidence of c-*erb*B-2 amplification and overexpression. These studies have shown statistically significant associations between amplification/overexpression and reduced overall and disease-free patient survival [108,162,199,247,250–259), overall survival alone [260–265], disease-free survival alone [266,267], as well as the lack of any association

Table 2. Retrospective studies reporting on incidence and prognostic significance of c-*erb*B-2 amplification and overexpression in breast cancer

Breast cancer incidence (%)		Patient survival[a] (+,− predictability)			
Oncogene amplification	Oncoprotein overexpression	*OS*	*DFS*	Correlated tumor parameter[a]	Reference
	31/185 (17%)	+	+	Grade, ER^-	253
6/61 (10%)	19/57 (33%)	NR	−		287
75/497 (15%)		+	NR	Grade	263
	104/465 (22%)	+	NR		264
	39/172 (23%)	−	+	S-phase, ER/PR^-	266
12/122 (10%)		−	−		268
	17/195 (9%)	−	−	Grade	272
	14/85 (16%)	+	+	Grade	256
20/89 (22%)		+	+	ER^-	257
	52/314 (17%)	+	+	Grade	258
	29/211 (14%)	+	+	Nodes, grade, ER/PR^-, S-phase	255
	278/1210 (23%)	+	NR	Size, grade	265
146/526 (28%)	51/187 (27%)	+	+		247
52/310 (17%)	68/360 (19%)	+	+	Size, ER/PR^-	250
16/48 (33%)	118/728 (16%)	+	+	Nodes, ER/PR^-	251
	62/292 (21%)	+	−	Grade	260
13/66 (20%)		+	−	Aneuploidy	261
8/53 (13%)		−	−	Nodes, size	161
17/157 (11%)		−	−		159
7/37 (19%)		+	+		162
10/57 (18%)	19/62 (31%)	+	−		262
	47/313 (15%)	+	+	Nodes, ER^-	252
19/77 (25%)	51/132 (39%)	+	+	PR^-, $p21^{ras}$	199
17/50 (34%)		−	−	PR^-, mitoses	269
	14/103 (14%)	−	−		270
	27/189 (14%)	−	−		271
28/176 (16%)	27/176 (15%)	+	+		108,163
	41/187 (22%)	+	+		259
	20/89 (23%)	NR	+		267
475/2437 (19%)	1099/5501 (20%)				

[a] Significant ($p < .05$) correlations reported between amplification or overexpression and reduced disease-free patient survival (DFS) or overall patient survival (OS), and the indicated tumor parameter. NR = not reported.

with patient outcome [159,161,268–272]. In some of these studies, the significant prognostic associations were observed only in subsets of axillary node-positive patients [108,163,247,250–252,258,263,265,266], poor nuclear grade tumors [260], or T3- and T4-stage primary tumors [252]. More recent studies have demonstrated an association with poor prognosis in node-negative patients as well [254,256,260,261]. One comparative analysis that focused only on node-negative patients found that a significantly greater number of relapsing patients had primary tumors with greater than or equal to threefold c-*erb*B-2 amplification; in this study a 70% recurrence predic-

tion was calculated for patients whose primary tumors had ≥6 c-*erb*B-2 gene copies [273]. Another study noted reduced overall survival for patients with ER-positive, node-negative, and c-*erb*B-2-overexpressing tumors [274].

The possibility that c-*erb*B-2 amplification or overexpression has a greater prognostic impact on overall patient survival rather than disease-free patient survival (Table 2) suggests that treatment following tumor relapse may be less effective on tumors bearing activated c-*erb*B-2. In support of this possibility, some in vitro models have predicted hormonal or cytotoxic resistance in c-*erb*B-2-overexpressing breast cancers [245]. As well, preliminary reports suggest an increased incidence of c-*erb*B-2-overexpressing tumors in patients relapsing on tamoxifen therapy [275,276].

Clinical indices that have been correlated significantly but inconsistently with amplified or overexpressed c-*erb*B-2 include higher histologic breast tumor grade [165,253–256,258,260,262,265,272,277], axillary nodal involvement [161,251,252,255,277–280], increased primary tumor size [250,265,271], younger patient age or premenopausal status at diagnosis [281], presence of inflammatory breast cancer [278,281], ER negativity [165,250–253,255, 257,266–279,282,283], PR negativity [165,199,250,251,255,266,269,278,282], high S-phase fraction [255,266,284], high mitotic activity [269], aneuploidy, and abnormal DNA content [166,255,285,286].

The above studies have many limitations, perhaps explaining their inconsistent conclusions. All have been retrospective in design and based on archived tumor samples, which introduces institutional and referral pattern biases as well as skewed patient study populations. Imperfect techniques for determining oncogene amplification and oncoprotein overexpression introduce variables that can be further confounded by the different cutoff values selected for assay positivity. Immunohistochemical results may depend on tissue fixation techniques and the type of antibody used [247], although some of these problems can be overcome [286].

Overall, there appears to be a very good correlation between c-*erb*B-2 amplification and overexpression [108,199,247,250,251,277,278,281,287–289], consistent with their nearly equivalent incidences, as shown in the accumulated database of Table 2. These two parameters are not equivalent, however, as also demonstrated [261]. Moreover, about 10–20% of overexpressing breast tumors appear to lack amplified c-*erb*B-2 gene copies [246,247,262,277,278,281,287,288], which supports the earlier suggestion that a c-*erb*B-2 transcriptional activator may be contributing to dysregulation of this proto-oncogene in some human breast cancers [234,235]. Disagreement about the clinical significance of c-*erb*B-2 amplification also stems from the failure to perform multivariate analyses in the majority of clinical studies reported to date. This form of analysis is important in order to determine if c-*erb*B-2 overexpression is codependent on other adverse prognostic markers. Ongoing prospective trials will help to determine whether c-*erb*B-2 amplification or overexpression represent clinically important markers with independent prognostic value.

Some insight into the biologic importance of c-*erb*B-2 in the induction and progression of human breast cancers has been gained from studies of surgical samples collected at various clinical stages of cancer progression, including normal mammary tissue, fibrocystic disease with hyperplasia, carcinoma in situ, invasive carcinoma, and late-stage metastatic lesions. Immunoreactive c-*erb*B-2 in tumor epithelium stains most intensely along the surface membrane, with weak cytoplasmic staining occasionally seen in the epithelial, myoepithelial, and vascular smooth muscle cells associated with normal and benign breast disease [270,290]. In contrast with the average 20% incidence of c-*erb*B-2 immunoreactivity among inverse cancers, in situ breast cancers demonstrate strong c-*erb*B-2 immunoreactivity in 29–42% of cases [271,291], with $\geq$80% of high-risk comedo-type ductal in situ lesions staining positive [271,285,291,292]. This immunoreactivity correlates with the high thymidine labelling index [291] and DNA content [285] of comedo-type in situ breast tumors. Porter et al. observed only rare c-*erb*B-2 positivity in either lobular carcinomas in situ or lobular invasive cancers [293]. Other histologic subtypes, such as cribiform and papillary forms of ductal in situ disease, stain positive for c-*erb*B-2 with a lower incidence, but this is not as well established due to variations in the pathologic subtyping of ductal in situ tumors [271,292]. For invasive breast cancers, c-*erb*B-2 expression seems largely restricted to ductal rather than lobular disease or less common histologic variants, including mucinous, medullary, tubular, cribiform, and papillary invasive cancers [294]. Concordance in c-*erb*B-2 positivity is observed between intraductal and invasive disease present within the same specimen, as well as between the invasive primary tumor and its nodal or distant metastatic sites in the same patient [163,270,196].

The recent finding of amplified c-*erb*B-2 gene copies occurring in purely ductal in situ tumors at twofold greater incidence than that found in primary invasive breast tumors has interesting implications that deserve further study. Liu et al. proposed that this finding could be explained by the existence of a 'protoinvasive' form of breast disease that is not morphologically recognized as in situ breast cancer, yet this form frequently progresses to c-*erb*B-2 negative invasive breast cancer [295]. Improvements in the genotyping of individual cells by techniques such as FISH should help determine which epithelial lesions first acquire a c-*erb*B-2 gain-of-function abnormality and thus constitute lesions with the highest risk of converting into invasive breast cancers.

Interest has also focused on developing therapeutic modalities that interfere with the transforming or growth-promoting function of overexpressed $p185^{erbB\text{-}2}$. Monoclonal antibodies to the rat *neu* product can inhibit the growth of *neu*-expressing cells in vitro as well as in vivo [296–299]. Active immunotherapy targeting the overexpressed $p185^{erbB\text{-}2}$ extracellular domain on tumor cells can inhibit and prevent the growth of breast tumors innoculated into syngeneic rats [300,301]. Likewise, antibodies to the human $p185^{erbB\text{-}2}$ extracellular domain have demonstrated antiproliferative effects both in

vitro and in vivo against overexpressing human breast cancer cell lines; in particular, muMAb4D5 not only inhibits $p185^{erbB\text{-}2}$-overexpressing tumors but also reverses the resistance of these cells to tumor necrosis factor-α [302,303]. There is additional evidence that such antibody therapy can potentiate the antitumor effects of certain chemotherapy agents such as cisplatin [304]. Antisense oligodeoxynucleotides have also been shown to decrease the expression of $p185^{erbB\text{-}2}$ and to inhibit the in vitro growth of overexpression cells [305]. Clinical trials with such novel anti-receptor therapeutics as muMAb4D5 are currently underway in patients with advanced breast and ovarian cancers. Bispecific and humanized (presumeably less immunogenic) versions of this therapeutic antibody are also ready for clinical testing.

*Coamplified genes and others related to c-*erb*B-2*

Amplification of nearby c-*erb*A1 has been noted in primary breast tumors, but this occurs exclusively in the presence of c-*erb*B-2 coamplification; however, the transcriptional expression of coamplified c-*erb*A1 has not been detected [108,166,271]. A gene homologous to c-*erb*B-2, termed c-*erb*B-3, has also been cloned. This gene is found on chromosome 12q13, is normally expressed in normal human epithelial tissue, and appears to be overexpressed in some breast cancer cell lines and primary breast tumors [306].

EGFR and other tyrosine kinase membrane receptors

The epidermal growth factor receptor, EGFR or $p175^{EGFR}$, is a membrane tyrosine kinase receptor with extensive homology to c-*erb*B-2. It may be classified as a proto-oncogene, since it is the cellular homologue of v-*erb*B, a transforming oncogene of the avian erythroblastosis virus that encodes a truncated EGFR-like protein that possesses constitutive kinase activity [reviewed in 307]. EGFR is normally expressed in many tissues and responds to locally produced growth factors necessary for growth and development during embryogenesis and wound healing [308]. Overexpression of EGFR has been detected in a variety of human cancers. Full malignant transformation by EGFR has not been demonstrated, although it mediates mitogenic cell stimulation in vitro by binding to one of its two known ligands, EGF or transforming growth factor-α (TGF-α) [309]. EGFR-transfected fibroblasts exhibit EGF-dependent morphologic transformation but they are unable to form tumors in nude mice [310]. TGF-α may stimulate tumor growth by interacting with EGFR as part of an autocrine loop, since TGF-α is secreted by about 50% of breast cancers (as well as normal breast tissue), and a significant fraction of these tumors also overexpress EGFR [311,312]. MMTV/TGF-α transgenic mice develop hyperplasia in breast alveoli and terminal ducts, followed by the stochastic appearance of breast adenomas and adenocarcinomas after pregnancy [313–315]. In murine

models, EGF deprivation by removal of the EGF-rich submandibular salivary gland reduces the incidence of breast tumors significantly, and this effect can be abrogated with the exogenous administration of EGF [316].

In human breast cancers, the amplification of EGFR has been seen in some established breast cancer cell lines but occurs in less than 5% of primary breast cancers [246,261,281]. Breast cancer overexpression of EGFR has been analyzed in a large number of reports in which it has been evaluated in relationship to patient prognosis and other tumor parameters. A recent review of 40 separate studies, including accumulated data on more than 5000 breast cancer cases, showed that EGFR overexpression occurs in about 40% of breast tumors [317]. Various investigators have found a correlation between EGFR overexpression and reduced overall and disease-free patient survival, both for node-positive and node-negative patient subgroups. In addition, overexpression correlates with decreased response to endocrine therapy in those patients with ER-positive metastatic disease [317], while there is no relationship between EGFR overexpression and the response to chemotherapy has been noted. Other tumor parameters consistently associated with EGFR overexpression include ER and PR negativity, nodal involvement and distant metastatic spread, ductal disease with poorly differentiated tumor histology, and DNA aneuploidy. Less consistently observed is an association between EGFR overexpression and tumor size, patient age, or menopausal status [317].

A correlation between EGFR and c-*erb*B-2 overexpression has been noted in two different reports [200,318], but this has been contradicted by four others [253,282,319,320]. However, the possibility of such a correlation raises the question of receptor cooperation or cross-signaling between EGFR and $p185^{erbB\text{-}2}$, leading to enhanced transforming potential. Cooperation has been documented in fibroblasts where full malignant transformation was induced by contransfected EGFR and c-*erb*B-2, but not with either proto-oncogene individually [321,322]. Two potential mechanisms for this interaction have been described. EGF, in an EGFR-dependent manner, can increase tyrosine phosphorylation of $p185^{erbB\text{-}2}$ [323]; as well, EGFR/$p185^{erbB\text{-}2}$ receptor heterodimers can potentially form that might be more resistant to intracellular internalization and downregulation [324]. Both of these mechanisms could lead to enhanced receptor tyrosine kinase activity and constitutive mitogenic signaling.

Insulin and insulinlike growth factors (IGF-I and IGF-II) appear to play a role in normal breast epithelial growth and metabolism, suggesting that abnormal interactions between these factors and their cognate receptors (IR, IGF-I-R, IGF-II-R) may also be important in autocrine or paracrine regulation of breast cancer growth. IGF-I and IGF-II are mitogens for breast cancer cells in vitro [325,326], and transcripts for both are detectable in most breast cancer specimens [325,327]. The IR and IGF-I-R are subclass II membrane tyrosine kinase receptors, while IGF-II-R (identical to the mannose-6-phosphate receptor) is composed of a single transmembrane

chain with a small intracellular domain that lacks tyrosine kinase activity [328–330]. All three receptors are expressed on both normal and malignant breast tissues [331–333]. Although considerable cross-reactivity takes place between these ligands and their receptors [334], the mitogenic effects of both IGF-I and IGF-II appear to be mediated by IGF-I-R [332], while the mitogenic effects of insulin occur via interaction of IR alone [332], or IR and IGF-I-R [335]. The overexpressed ligands may function in a paracrine fashion in which normal stromal cells are recruited by malignant breast epithelial cells into overexpressing either IGF-I or IGF-II [327,336].

Papa et al. demonstrated a sixfold higher mean IR content in 159 breast cancer specimens compared to normal breast tissue and showed that the overexpressed IR is functional and confined to the malignant breast cells as opposed to admixed stromal and inflammatory cells [333]. In this study there was a significant correlation between IR overexpression and tumor size, histologic grading, and estrogen receptor content. The observations and mechanisms related to overexpression of IR are discussed in further detail in Chapter 9. Pekonen et al. similarly demonstrated increased IGF-I-R in breast cancers as compared to normal breast tissue [331]; more recently, Bonneterre et al. studied 297 patients and found that elevated IGF-I-R expression correlates with increased disease-free and overall survival in node-positive patients only, and is associated with increased tumor differentiation and ER and PR positivity [337]. In another series, prognostic correlation with IGF-I-R overexpression was not seen [338]. Altogether, these findings suggest that abnormalities in insulin and related factors, as well as their receptor systems, may play a biologic role in the development or progression of breast cancer, although the question of whether these are primary or secondary events in breast tumorigenesis remains to be clarified. Experimental models indicate that IR overexpression can confer ligand-dependent transformation in fibroblasts [339], but there is not yet definitive evidence that this transforming mechanism is operative in the clinical development of breast cancer.

It is also possible that various tyrosine kinase receptors, including $p185^{erbB\text{-}2}$, EGFR, IR, and IGF-I-R, play a combined or cooperative role in promoting or maintaining breast cancer growth. In support of their combined biologic potential, there is evidence that total tyrosine kinase specific activity (membrane and cytosolic) is considerably higher in breast cancer tissues as compared to normal breast tissue or benign breast tumors; furthermore, this high total tyrosine kinase activity appears to be associated with worse patient prognosis [340].

Lastly, other membrane peptide receptors may influence the growth-promoting autocrine or paracrine loops that utilize tyrosine kinase receptors, suggesting new therapeutic modalities for breast cancer. For example, somatostatin is a widely distributed peptide that binds to its own receptor and suppresses the release of various pituitary and gastrointestinal hormones, yet it can also decrease EGF and IGF-I secretion [341] and directly inhibit

breast cancer cell growth in vitro [342]. Foekens et al. noted an increased disease-free survival in the 15% of patients whose breast cancers express the somatostatin receptor [338], introducing further rationale for the clinical testing of somatostatin analogues as therapy for breast cancer.

The oncogenic potential of steroid receptors

The concept of structurally altered steroid receptors functioning as oncoproteins has recently been proposed [reviewed in 343]. The steroid/thyroid receptor superfamily is a class of ligand-dependent DNA-binding transactivating proteins [reviewed in 344]. The v-*erb*A transforming oncogene of the avian erythroblastosis virus encodes a truncated analogue of the mammalian thyroid hormone receptor that acts as a dominant-negative transcriptional repressor of genes normally activated by the thyroid-hormone-bound thyroid receptor [345]. As these genes appear to be involved in normal tissue differentiation, the repressive effect of v-*erb*A or a similarly truncated member of the receptor superfamily may be oncogenic. Such receptor abnormalities may occur by either genetic or epigenetic mechanisms. For example, a structurally rearranged retinoic acid receptor-α (RAR-α) gene resulting from a t(15;17) chromosomal translocation in acute promyelocytic leukemia generates a new fusion gene product between portions of the PML and RAR-α genes; this DNA-binding fusion protein appears to confer and propagate the transformed phenotype because of its abnormal DNA binding and interaction with other transcription factors [346,347]. In fact, treatment with excess *trans*-retinoic acid induces clinical remissions in patients with acute promyelocytic leukemia, presumably by binding and preventing the abnormal transactivating function of this fusion protein [348].

Recent work in human breast cancers has focused on ER variants that may confer estrogen-independent or constitutive DNA binding and abnormal gene transactivation, leading to a more aggressive breast cancer phenotype [349,350]. In a murine model, ER-positive mammary tumors that lose estrogen dependence with serial transplantation exhibit a gradual increase in truncated ER isoforms by an apparent epigenetic mechanism [351]. Fuqua et al. have examined the infrequent subgroup of human breast cancers that are ER negative/PR positive, postulating that the ER in these tumors is structurally altered and immunologically undetectable, yet still able to bind DNA and transcriptionally activate the PR gene [352]. These investigators have identified potential ER variants produced by abnormal RNA splicing that lack key amino acid sequences in the hormone binding domain. In a different approach, Scott et al. used gel retardation assays to show that many ER-positive human breast tumors appear to contain either truncated forms of DNA-binding ER or unusual forms of immunoreactive ER that are unable to bind DNA [353]. An ER variant lacking exon 7 has been reported in ER^+/PR^- breast cancers that can interfere with wild-type

ER DNA binding in a dominant negative manner [354]. While continued investigations in this area will no doubt clarify how abnormal ER can contribute to a more malignant breast cancer phenotype, there has been little effort to determine whether other steroid receptor abnormalities potentially contribute to breast cancer development or progression.

Other functional abnormalities associated with breast cancer invasion and metastasis

There is increasing interest in the molecular mechanisms that account for breast tumor invasion and metastasis. The elaboration of Cathepsins B and D, urokinase-type plasminogen activator, and collagenase type IV are all early events in the breakdown of extracellular matrix necessary for mammary epithelial cell invasion. Increased levels of these proteinases are known to correlate with poor prognosis in breast cancer patients [355 and references therein, reviewed in 356], but the genetic controls regulating expression of the many known breast cancer proteinases and proteinase inhibitors remain poorly understood. A new metalloproteinase gene, stromolysin-3, was recently identified by a subtractive hybridization technique using human breast carcinoma and breast adenoma specimens as tissue sources [357]. Unexpectedly, this tumor associated proteinase was found to be stromally produced, presumably induced by paracrine factors arising from adjacent breast cancer cells.

The putative metastasis supressor gene, *nm23*, was initially identified by Steeg et al. (see Chapter 18), also using a subtractive hybridization technique [358]. The gene is virtually identical to the *Drosophila awd* developmental gene for abnormal wing discs [359] and is homologous to the phylogenetically conserved enzyme, nucleoside diphosphate kinase [360], raising the possibility that a G-protein-related pathway is involved in the regulation of tumor metastasis. Transfection of the *nm23* gene into a melanoma cell line reduces its metastatic potential [361]. In breast cancer, significantly lower *nm23* mRNA levels have been measured in the tumors of patients with increased lymph node involvement [362]; and loss of *nm23* mRNA appears to be associated with reduced disease-free and overall patient survival [363]. It is still not clear if *nm23* loss is a sufficient or necessary step in developing a metastatic phenotype. As discussed earlier, the putative *nm23* tumor suppressor gene is localized to chromosome 17p11-q11 and shows allelic deletion in 16 of 25 (64%) breast cancer specimens [69]. Another highly homologous gene, designated *nm23*-H2, has recently been cloned, and preliminary analysis of breast tumors with this gene probe does not show a correlation between decreased expression and metastatic potential [364].

Searches for similar breast cancer suppressor genes using the same technique of subtractive hybridization, but employing short-term normal breast epithelial cell cultures and breast cancer cell lines have yielded clones for

genes encoding the gap junction protein connexin 26, a calcium-binding protein of the S-100 family, members of the keratin gene family, the detoxifying enzyme glutathione-S-transferase π, and a bone matrix (gla) protein [365,366]. Although these results must be considered tentative given the artifact that can be introduced by this technique, it is interesting to note that these tumor-associated if not tumor-suppressing genes appear to affect critical cell activities, such as intercellular communication and intracellular signaling, xenobiotic detoxification, and cell differentiation, all of which are functionally abnormal in the malignant phenotype.

Summary

Cytogeneticists first proposed that the karyotypic abnormalities identified on chromosomes 1, 3, 6, 11, 13, 16, 17, and 18 supported a genetic basis for breast cancer. Such abnormal banding patterns, however, may represent either loss-of-function or gain-of-function molecular events. RFLP analyses have since confirmed that 20–60% of primary and spontaneous human breast tumors exhibit allelic losses on these same chromosomes, although the exact genes involved at these chromosomal sites remain largely unknown. Knowledge gained about the *Rb*-1 and p53 tumor suppressor genes at 13q14 and 17p13 in breast and other human tumors supports the paradigm that for any chromosomal locus, allelic loss associated with a mutation in the remaining tumor allele signifies an involved tumor suppressor gene. Given this paradigm, there are nearly a dozen putative breast tumor suppressor genes under active investigation, with most investigators now focusing on various chromosome 17 loci.

Among the known proto-oncogenes found activated in breast cancer, amplification of c-*erb*B-2 at 17q21 is the most widely studied and clinically significant gain-of-function event uncovered to date, occurring in about 20% of all primary breast tumors. The involvement of this overexpressed membrane receptor has engendered interest in related tyrosine kinase receptors, such as EGFR, IR, and IGF-I-R, as well as their respective ligands, which may be overexpressed in a greater fraction of tumors, contributing to the autocrine and paracrine regulation of breast cancer growth and metastasis. New attention is being given to the potentially oncogenic function of structurally altered nuclear transactivating steroid hormone receptors, such as ER, whose overexpression has long been used to determine endocrine therapy and prognosis for individual breast cancer patients. While c-*myc* was one of the first known proto-oncogenes to be found amplified and overexpressed in human breast cancers, the actual incidence and clinical significance of its activation remain disputed and in need of further study. Lastly, we can expect greater clarification about the importance of various 11q13 genes found coamplified in nearly 20% of primary breast cancers, and pursuit into the intriguing possibility that a

cyclin-encoding gene represents the overexpressed locus of real interest in this amplicon.

Virtually all of these important genetic abnormalities identified thus far are associated with but not restricted to human breast cancers. The absence of identifiable molecular defects relating to the tissue specificity of this malignancy must be considered a substantial gap in our basic understanding of breast carcinogenesis. Further clues may come with a better understanding of genetic abnormalities potentially associated with the commonly ascribed breast cancer risk factors that include familial predisposition, dietary fat intake, and endogenous sex steroid levels.

References

1. Hainsworth PJ, Raphael KL, Stillwell RG, Bennett RC, Garson OM: Cytogenetic features of twenty-six primary breast cancers. Cancer Genet Cytogenet 53:205–218, 1991.
2. Trent JM: Cytogenetic and molecular biologic alterations in human breast cancer: A review. Breast Cancer Res Treat 5:221–229, 1985.
3. Heim S, Mitelman F: Cancer Cytogenetics. Alan R. Liss, New York, 1987, pp 232–233.
4. Mitelman F: Catalog of Chromosome Aberrations in Cancer, 3rd ed. Alan R. Liss, New York, 1988.
5. Ferti-Passantonopoulou A, Panani AD, Raptis S: Preferential involvement of 11q23–24 and 11p15 in breast cancer. Cancer Genet Cytogenet 51:183–188, 1991.
6. Dutrillaux B, Gerbault-Sereau M, Zafrani B: Characterization of chromosomal anomalies in human breast cancer. A comparison of 30 paradiploid cases with few chromosome changes. Cancer Genet Cytogenet 49:203–217, 1990.
7. Gerbault-Sereau M, Vielh P, Dutrillaux B: Cytogenetic study of twelve near-diploid breast cancers with chromosomal changes. Annales Genetique 30:146–151, 1987.
8. Gebhart E, Brudelein S, Augustus M, Seibert E, Feldner J, Schmidt W: Cytogenetic studies on human breast carcinomas. Breast Cancer Treat Res 8:125–138, 1986.
9. Saint-Ruf C, Gerbault-Sereau M, Viegas-Pequignot E, Zafrani B, Malfoy B, Dutrillaux B: Recurrent homogenously staining regions in 8p1 in breast cancer and lack of amplification of POLB, LHRH and PLAT genes. Cancer Genet Cytogenet 52:27–35, 1991.
10. Band V, Zajchowski D, Stenman G, Morton CC, Kulesa V, Connolly J, Suger R: A newly established metastatic breast tumor cell line with integrated amplified copies of *erb*B-2 and double minute chromosomes. Genes Chromosomes Cancer 1:48–58, 1989.
11. Saint-Ruf C, Gerbault-Seureau M, Viegas-Pequiqnot E, Zafrani B, Cassingena R, Dutrillaux B: Proto-oncogene amplification and homogeneously staining regions in human breast carcinomas. Genes, Chromosomes Cancer 2:18–26, 1990.
12. Sato T, Akiyama F, Sakamoto G, Kasumi F, Nakamura Y: Accumulation of genetic alterations and progression of primary breast cancer. Cancer Res 51:5794–5799, 1991.
13. Genuardi M, Tsihira H, Anderson DE, Saunders GF: Distal deletion of chromosome 1p in ductal carcinoma of the breast. Am J Human Genet 45:73–82, 1989.
14. Mathew CGP, Smith BA, Thorpe K, Wong Z, Royle NJ, Jeffreys AL, Ponder BAJ: Deletions of genes on chromosome 1 in endocrine neoplasia. Nature 328:524–526, 1987.
15. Mathew CGP, Chin KS, Easton DF, Thorpe K, Carter C, Liou GI, Fong S-L: A linked genetic marker for multiple endocrine neoplasia type 2a on chromosome 10. Nature 328:527–528, 1987.
16. Simpson NE, Kidd KK, Goodfellow PJ, McDermid H, Myers S, Kidd JR, Jackson CE, Duncan AM, Farrer LA, Brasch K: Assignment of multiple endocrine neoplasia type 2A to chromosome 10 by linkage. Nature 328:528–530, 1987.

17. Chen L-C, Dollbaum C, Smith HS: Loss of heterozygosity on chromosome 1q in human breast cancer. Proc Natl Acad Sci USA 86:7204–7207, 1989.
18. Devilee P, van Vliet M, Bardoel A, Kievits T, Kuipers-Dijkshoorn N, Pearson PL, Cornelisse CJ: Frequent somatic imbalance of marker alleles for chromosome 1 in human primary breast carcinoma. Cancer Res 51:1020–1025, 1991.
19. Gendler S, Taylor-Papadimitriou J, Duhig T, Rothbard J, Burchell J: A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. J Biol Chem 263:12820–12823, 1988.
20. Siddiqui J, Abe M, Hayes D, Sharri E, Yunis E, Kufe DW: Isolation and sequencing of a cDNA coding for the human DF-3 breast carcinoma-associated antigen. Proc Natl Acad Sci USA 85:2320–2323, 1988.
21. Merlo GR, Siddiqui JM, Cropp CS, Liscia DS, Lidereau R, Callahan R, Kufe DW: Frequent alteration of the DF3 tumor-associated antigen gene in primary human breast carcinomas. Cancer Res 49:6966–6971, 1989.
22. Gendler SJ, Cohen EP, Craston A, Taylor-Papadimitriou J, Johnstone G: Modifications of chromosome I in breast cancer. Proc Am Assoc Cancer Res 30:39, 1989 (abstr).
23. Chen L-C, Ngo JT, Kurisu W, Smith HS: Loss of heterozygosity on chromosome 1q and 17p in human breast cancer (abstr #1947). Proc Am Assoc Cancer Res 31:328, 1990.
24. Kufe DW, Inghirami G, Abe M, Hayes D, Justi-Wheeler H, Schlom J: Differential reactivity of a novel monoclonal antibody (DF3) with human malignant versus benign breast tumors. Hybridoma 3:223–232, 1984.
25. Lundy J, Thor A, Maenza R, Schlom J, Forourar F, Testa M, Kufe D: Monoclonal antibody DF3 correlates with tumor differentiation and hormone receptor status in breast cancer patients. Breast Cancer Res Treat 5:269–276, 1985.
26. Hayes DF, Mesa-Tejada R, Papsidero LD, Croghan GA, Korzun AW, Norton L, Strauchen JA, Grimes M, Weiss RB, Ree HJ, Thor AD, Koerner FC, Rice MA, Barcos M, Kufe DW. Prediction of prognosis in primary breast cancer by detection of a high molecular weight mucus-like antigen using monoclonal antibodies DF3, F36/22, and CU18: A Cancer and Leukemia Group B study. J Clin Oncol 9:1113–1123, 1991.
27. Ali IU, Lidereau R, Callahan R: Presence of two members of c-*erb*A receptor gene family (c-*erb*Aβ and c-*erb*A2) in smallest region of somatic homozygosity on chromosome 3p21–25 in human breast cancer. J Natl Cancer Inst 81:1815–1820, 1989.
28. Devilee P, Van der Broek M, Kuipers-Dijkshoorn N, Kolluri R, Kahn PM, Pearson PL, Cornelisse CF: At least four different chromosomal regions are involved in loss of heterozygosity in human breast carcinoma. Genomics 5:554–560, 1989.
29. Devilee P, van Vliet M, van Sloun P, Kiupers-Dijkshoorn NK, Hermans J, Pearson PL, Cornelisse CJ: Allelotype of human breast carcinoma: A second major site for loss of heterozygosity is on chromosome 6q. Oncogene 6:1705–1711, 1991.
30. Ali IU: unpublished.
31. Naylor SL, Johnson BE, Minna JD, Sakaguchi, AY: Loss of heterozygosity of chromosome 3p markers in small-cell lung cancer. Nature 329:454, 1987.
32. Kovacs G, Erlandsson R, Boldog F, Ingvarsson S, Muller-Brechlin R, Klein G, Sumegi J: Consistent chromosome 3p deletion and loss of heterozygosity in renal cell carcinoma. Proc Natl Acad Sci USA 85:1571–1575, 1988.
33. Seizinger BR, Rouleau GA, Ozelius LJ, Lane AH, Farmer GE, Lamiell JM, Haines J, Yuen JW, Collins D, Majoor-Krakauer D: Von Hippel-Lindau disease maps to the region of chromosome 3 associated with renal cell carcinoma. Nature 332:268–269, 1988.
34. Zenke M, Muñoz A, Sap J, Vennstrom B, Beug H: v-*erb*A oncogene activation entails the loss of hormone-dependent regulator activity of c-*erb*A. Cell 61:1035–1049, 1990.
35. Bieche I, Champeme MH, Matifas F, Hacene K, Callahan R, Lidereau R: Loss of heterozygosity on chromosome 7q and aggressive primary breast cancer. Lancet 339:139–143, 1992.
36. Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF, Aaronson SA: Identification of the hepatocyte growth factor receptor as the c-*met* proto-

oncogene product. Science 251:802–804, 1991.
37. Mackay J, Elder PA, Porteous DJ, Steel CM, Hawkins RA, Going JJ, Chetty U: Partial deletion of chromosome 11p in breast cancer correlates with size of primary tumour and oestrogen receptor level. Br J Cancer 58:710–714, 1988.
38. Ali UI, Lidereau R, Theillet C, Callahan R: Reduction to homozygosity of genes on chromosome II in human breast neoplasia. Science 238:185–188, 1987.
39. Barkardottir RB, Johannsson OT, Arason A, Gudnason V, Egilsson V: Polymorphism of the c-Ha-*ras*-1 proto-oncogene in sporadic and familial breast cancer. Int J Cancer 11: 251–255, 1989.
40. Sheng ZM, Guerin M, Gabillot M, Spellmann M, Riou G: c-Ha-*ras*-1 polymorphs in human breast carcinomas: Evidence for a normal distribution of allele. Oncogene Res 2:245–250, 1988.
41. Hall JM, Huey B, Morrow J, Newman B, Lee M, Jones E, Carter C, Buehring GC, King M-C: Rare HRAS alleles and susceptibility to human breast cancer. Genomics 6:188–191, 1990.
42. Theillet C, Lidereau R, Escot C, Hutzell P, Brunet M, Gest J, Schlom J, Callahan R: Loss of a c-H-*ras*-1 allele and aggressive human primary breast carcinomas. Cancer Res 46:4776–4781, 1986.
43. Lundberg C, Skoog L, Cavenee WK, Nordenskjold M: Loss of heterozygosity in human ductal breast tumors indicates a recessive mutation on chromosome 13. Proc Natl Acad Sci USA 84:2372–2376, 1987.
44. Lee E Y-HP, To H, Shew J-Y, Bookstein R, Skully P, Lee W-H: Inactivation of the retinoblastoma susceptibility gene in human breast cancers. Science 241:218–221, 1988.
45. T'Ang A, Varley JM, Chakraborty S, Murphree AL, Fung Y-KT: Structural rearrangement of retinoblastoma gene in human breast carcinoma. Science 242:263–272, 1988.
46. Varley JM, Armour J, Swallow JE, Jeffreys AF, Ponder BAJ, T'Ang A, Fung Y-KT, Brammar WJ, Walker RA: The retinoblastoma gene is frequently altered leading to loss of expression in primary tumors. Oncogene 4:425–429, 1989.
47. Hartley AL, Birch JM, Marsden HB, Harris M: Breast cancer risk in mothers of children with osteosarcoma and chondrosarcoma. Br J Cancer 54:819–823, 1986.
48. Sato T, Tanigami A, Yamakawa K, Akiyama F, Kasumi F, Sakamoto G, Nakamura Y: Allelotype of breast cancer: Cumulative allele losses promote tumor progression in primary breast cancer. Cancer Res 50:7184–7189, 1990.
49. Hollstein M, Sidranski D, Vogelstein B, Harris CC: p53 mutations in human cancers. Science 253:49–53, 1991.
50. Devilee P, Cornelisse CJ, Kuipers-Dijkshoorn N, Jonkers C, Pearsol PL: Loss of heterozygosity on 17p in human breast carcinomas: Defining the smallest common region of deletion. Cytogenet Cell Genet 53:52–54, 1990.
51. Chen L-C, Neubauer A, Kurisu W, Waldman FM, Ljung B-M, Goodson W 3rd, Goldman ES, Moore D II, Balaz M, Liu E, Mayall BM, Smith HS: Loss of heterozygosity on the short arm of chromosome 17 is associated with a high proliferative capacity and DNA aneuploidy in primary breast cancer. Proc Natl Acad Sci USA 88:3847–3851, 1991.
52. Crawford LV, Pim DC, Bulbrook RD. Detection of antibodies against the cellular protein p53 in sera from patients with breast cancer. Int J Cancer 30:403–408, 1982.
53. Crawford LV, Pion DC, Lamb P: The cellular protein p53 in human tumors. Mol Biol Med 2:261–272, 1984.
54. Thor AD, Moore DH II, Edgerton SM, Kawasaki ES, Reihaus E, Lynch HT, Marcus JM, Schwartz L, Chen LC, Mayall BM, Smith HS: Accumulation of p53 tumor suppressor protein: an independent marker of prognosis in breast cancer. J Natl Cancer Inst 84: 845–855, 1992.
55. Davidoff AM, Herndon JE 2nd, Glover NS, Kerns BJ, Pence JC, Iglehart JD, Marks JR: Relation between p53 overexpression and established prognostic factors in breast cancer. Surgery 110:259–264, 1991.
56. Iwaya K, Tsuda H, Hiraide H, Tamaki K, Tamakuma S, Fukutomi T, Mukai K,

Hirohashi S: Nuclear p53 immunoreaction associated with poor prognosis of breast cancer. Jpn J Cancer Res 82:835–840, 1991.
57. Cattoretti G, Rilke F, Andreola S, D'Amato L, Delia D: p53 expression in breast cancer. Int J Cancer 41:178–183, 1988.
58. Ostrowski JL, Sawan A, Henry L, Wright C, Henry JA, Hennessy C, Lennard TJ, Angus B, Horne CH: p53 expression in human breast cancer related to survival and prognostic factors: An immunohistochemical study. J Pathol 164:75–81, 1991.
59. Davidoff AM, Humphrey PA, Iglehart FD, Marks JR: Genetic basis for p53 overexpression in human breast cancer. Proc Natl Acad Sci USA 88:5006–5010, 1991.
60. Bartek J, Bartkova J, Vojtesek B, Staskova Z, Rejthar A, Kovanik J, Lane DP: Patterns of expression of the p53 tumor suppressor in human breast tissues and tumors in situ and in vitro. Int J Cancer 46:839–844, 1990.
61. Davidoff AM, Kerns BJ, Iglehart JD, Marks JR: Maintenance of p53 alterations throughout breast cancer progression. Cancer Res 51:2605–2610, 1991.
62. Bartek J, Iggo R, Gannon J, Lane DP: Genetic and immunochemical analysis of resistant p53 in human breast cancer cell lines. Oncogene 5:893–899, 1990.
63. Prosser J, Thompson AM, Cranston G, Evans HF: Evidence that p53 behaves as a tumor suppressor gene in sporadic breast tumors. Oncogene 5:1573–1579, 1990.
64. Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P, Glover T, Collins FS, Weston A, Modali R, Harris CC, Vogelstein B: Mutations in the p53 gene occur in diverse human tumors. Nature 342: 705–708, 1989.
65. Coles C, Thompson AM, Elder PA, Cohen BB, MacKenzie IM, Cranston G, Chetty U, Mackay J, MacDonald M, Nakamura Y, Hoyheim B, Steel CM: Evidence implicating at least two genes on chromosome 17p in breast carcinogenesis. Lancet 336:763–765, 1990.
66. Runnebaum IB, Nagarajan M, Bowman M, Soto D, Sukumar S: Mutations in p53 as potential molecular markers for human breast cancer. Proc Natl Acad Sci USA 88: 10657–10661, 1991.
67. Malkin D, Li FP, Strong LC, Fraumeni JF, Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA, Friend SH: Germ line p53 mutation in a familial syndrome of breast cancer sarcomas and other neoplasms. Science 250:1233–1238, 1990.
68. Srivastava S, Zou Z, Pirollo K, Blattner W, Chang EH: Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni Syndrome. Nature 348: 747–749, 1990.
69. Leone A, McBride OW, Weston A, Wang MC, Anglard P, Cropp CS, Goepel JR, Lidereau R, Callahan R, Steeg PS: Somatic allelic deletion of nm23 in human cancer. Cancer Res 51:2490–2493, 1991.
70. Hall JF, Lee MK, Newman B, Morrow JE, Anderson LA, Huey B, King M-C: Linkage of early-onset familial breast cancer to chromosome 17q21. Science 250:1684–1689, 1990.
71. Narod SA, Feunteun J, Lynch HT, Watson P, Conway T, Lynch J, Lenoir GM: Familial breast-ovarian cancer locus on chromosome 17q12–23. Lancet 338:82–83, 1991.
72. Poutanen M, Moncharmont B, Vihko R: 17β-hydroxysteroid dehydrogenase gene expression in human breast cancer cells: Regulation of expression by a progestin. Cancer Res 52:290–294, 1992.
73. Fearon ER, Cho KR, Nigro JM, Vogelstein B: Identification of a chromosome 18q gene that is altered in colorectal cancers. Science 247:49–50, 1990.
74. Devilee P, van Vliet M, Kiupers-Dijkshoorn N, Pearson PL, Cornelisse CJ: Somatic genetic changes on chromosome 18 in breast carcinomas: Is the DCC gene involved? Oncogene 6:311–315, 1991.
75. Nusse R, Varmus HE: Many tumors induced by mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 31:99–109, 1982.
76. Dickson C: Role of the *Int*-genes in murine mammary tumor development and implications for human breast cancer. Int J Cancer 5(Suppl.):51–54, 1990.
77. Nusse R, Brown A, Papkoff J, Scambler P, Shackleford G, McMahon A, Moon R,

Varmus H: A new nomenclature for *int*-1 and related genes: The *Wnt* gene family. Cell 64:231, 1991.
78. Rijsewijk F, Schuermann M, Wagenaar E, Parren P, Weigel D, Nusse R: The Drosophila homologue of the mouse mammary oncogene *int*-1 is identical to the segment polarity gene *wingless*. Cell 50:649–657, 1987.
79. Wilkinson DG, Bailes AJ, McMahon AP: Expression of the proto-oncogene *int*-1 is restricted to specific neural cells in the developing mouse embryo. Cell 50:79–88, 1987.
80. Rijsewijk F, Van Deemter L, Wagemaar E, Sonnenberg A, Nusse R: Transfection of the *int*-1 mammary oncogene in cuboidal RAC mammary cell line results in morphological transformation and tumorigenicity. EMBO J 6:127–131, 1987.
81. Tsukamoto AS, Grosschedl R, Guzman RC, Varmus HE: Expression of the *int*-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell 55:619–625, 1988.
82. Jakobovitz A, Shackleford GM, Varmus HE, Martin GR: Two proto-oncogenes implicated in mammary carcinogenesis, *int*-1 and *int*-2, are independently regulated during mouse development. Proc Natl Acad Sci USA 83:7806–7810, 1986.
83. Wilkinson DG, Peters G, Dickson C, McMahon AP: Expression of the FGF-related proto-oncogene *int*-2 during gastrulation and neurulation in the mouse. EMBO J 7: 691–695, 1988.
84. Wilkinson DG, Bailes AJ, McMahon AP: Expression of the FGF-related proto-oncogene *int*-2 suggests multiple roles in fetal development. Development 105:131–136, 1989.
85. Represa J, Leon Y, Miner C, Giraldez F: The *int*-2 proto-oncogene is responsible for induction of the inner ear. Nature 353:561–563, 1991.
86. Dickson C, Peters G: Potential oncogene products related to growth factors. Nature 326:833, 1987.
87. Muller WJ, Lee FS, Dickson C, Peters G, Pattengale P, Leder P: The *int*-2 gene product acts as an epithelial growth factor in transgenic mice. EMBO J 9:907–913, 1990.
88. Peters G, Lee AE, Dickson C: Activation of cellular gene by mouse mammary tumour virus may occur early in mammary tumour development. Nature 309:273–275, 1984.
89. Nusse R: The int genes in mammary tumorigenesis and in normal development. Trends Genet 4:291–295, 1988.
90. Morris VL, Rao TR, Kozak CA, Gray DA, Lee Chan ECM, Cornell TJ, Taylor CB, Jones RF, McGrath CM: Characterization of *Int*-5, a locus associated with early events in mammary carcinogenesis. Oncogene Res 6:53–63, 1991.
91. Gallahan D, Callahan A: Mammary tumorigenesis in feral mice: Identification of a new *int* locus in mouse mammary tumor virus (Czech II)-induced mammary tumors. J Virol 61:66–74, 1987.
92. Sakamoto H, Mori M, Taira M, Yoshida T, Matsukawa S, Shimizu K, Sekuguchi M, Terada M, Sugimura T: Transforming gene from human stomach cancers and a noncancerous portion of stomach mucosa. Proc Natl Acad Sci USA 83:3997–4001, 1986.
93. Delli Bovi P, Curatola AM, Kern FG, Greco A, Ittman M, Basilico C: An oncogene isolated by transfection of Kaposi's sarcoma DNA encodes a growth factor that is a member of the FGF family. Cell 50:729–737, 1987.
94. Peters G, Brookes S, Smith R, Placzek M, Dickson C: The mouse homolog of the *hst/k*-FGF gene is adjacent to *int*-2 and is activated by proviral insertion in some virally induced mammary tumors. Proc Natl Acad Sci USA 86:5678–5682, 1989.
95. Yoshida T, Miyogawa K, Odagiri H, Sakamoto H, Little PF, Terada M, Sugimura T: Genomic sequence of *hst*, a transforming gene encoding a protein homologous to fibroblast growth factors and the *int*-2 encoded protein. Proc Natl Acad Sci USA 84: 7305–7309, 1987. (Published erratum appears in Proc Natl Acad Sci USA 85:1967, 1988)
96. Peters G, Lee AE, Dickson C: Concerted activation of two potential proto-oncogenes in carcinomas induced by mouse mammary tumor virus. Nature 320:628–631, 1986.
97. Peters G, Kozak C, Dickson C: Mouse mammary tumor virus integration regions *int*-1 and *int*-2 map on different mouse chromosomes. Mol Cell Biol 4:375–378, 1984.

98. Tsuda T, Tahara E, Kajiyama G, Sakamoto H, Terada M, Sugimura T: High incidence of coamplification of *hst*-1 and *int*-2 genes in human esophageal carcinomas. Cancer Res 49:5505–5508, 1989.
99. Zhou DJ, Casey G, Cline MJ: Amplification of human *int*-2 in breast cancers and squamous carcinomas. Oncogene 2:279–282, 1988.
100. Tsutsumi M, Sakamoto H, Yoshida T, Kakizoe T, Koiso K, Sugimura T, Terada M: Coamplification of the *hst*-1 and *int*-2 genes in human cancers. Jpn J Cancer Res 79: 428–432, 1988.
101. Hatada I, Tokino T, Ochiya T, Matsubara K: Co-amplification of integrated hepatitis B virus DNA and transforming gene *hst*-1 in a hepatocellular carcinoma. Oncogene 3: 537–540, 1988.
102. Adelaide J, Mattei MG, Marics I, Raybaud F, Planche J, deLapeyriere O, Birnbaum D: Chromosomal localization of the *hst* oncogene and its co-amplification with the *int*-2 oncogene in a human melanoma. Oncogene 2:413–416, 1988.
103. Ali IU, Merlo G, Callahan R, Lidereau R: The amplification unit on chromosome 11q13 in aggressive primary breast tumors entails the *bcl*-1, *int*-2 and *hst* loci. Oncogene 4: 89–92, 1989.
104. Fantl V, Brookes S, Smith R, Casey G, Barnes D, Johnstone G, Peters G, Dickson C: Characterization of the proto-oncogene *INT*-2 and its potential for diagnosis of human breast cancer. In: Furth M, Greaves M (eds): Cancer Cells, Vol. 7: Molecular Diagnosis of Human Cancer. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989, pp 283–287.
105. Theillet C, LeRoy X, deLapeyriere O, Grosgeorges J, Adnane J, Reynaud S, Simony-Lafontaine J, Goldfarb M, Escot C, Birnbaum D, Gaudray P: Amplification of FGF-related genes in human tumors: Possible involvement of *HST* in breast carcinomas. Oncogene 4:915–922, 1989.
106. Lidereau R, Callahan R, Dickson C, Peters G, Escot C, Ali IU: Amplification of the *int*-2 gene in primary human breast tumors. Oncogene Res 2:285–291, 1988.
107. Varley JM, Walker RA, Casey G, Brammar WJ: A common alteration of the *int*-2 proto-oncogene in DNA from primary breast carcinomas. Oncogene 3:87–91, 1988.
108. Tsuda H, Hirohashi S, Shimosato Y, Hirota T, Tsugane S, Yamamoto H, Miyajima N, Toyoshima K, Yamamoto T, Yokota J, Yoshida T, Sakamoto H, Terada M, Sugimura T: Correlation between long-term survival in breast cancer patients and amplification of two putative oncogene-coamplification units: *hst*-1/*int*-2 and c-*erb*B-2/*ear*-1. Cancer Res 49:3104–3108, 1989.
109. Adane J, Gaudray P, Simon M-P, Simony-Lafontaine J, Jeanteur P, Theillet C: Proto-oncogene amplification and human breast tumor phenotype. Oncogene 4:1389–1395, 1989.
110. Nguyen C, Roux D, Mattei MG, deLapeyriere O, Goldfarb M, Birnbaum D, Jordan B: The FGF-related oncogenes *hst* and *int*-2 and the *bcl*-1 locus are contained within one megabase in band q13 of chromosome 11, while the *fgf*-5 oncogene maps to 4q21. Oncogene 3:703–708, 1988.
111. Wada A, Sakamoto H, Katoh O, Yoshida T, Yokota J, Little PFR, Sugimura T, Terada M: The homologous oncogenes, *HST*1 and *INT*2, are closely located in the human genome. Biochem Biophys Res Commun 157:828–835, 1988.
112. Machotka SV, Garrett CT, Schwantz AM, Callahan R: Amplification of the protooncogenes *int*-2, c-*erb*B-2 and c-*myc* in human breast cancer. Clin Chim Acta 184:207–217, 1989.
113. Meyers SL, O'Brien MT, Smith T, Dudley JP: Analysis of the *int*-1, *int*-2, c-*myc* and *neu* oncogenes in human breast carcinomas. Cancer Res 50:5911–5918, 1990.
114. Liscia DS, Merlo GR, Garrett C, French D, Mariani-Constantini R, Callahan R: Expression of *int*-2 mRNA in human tumors amplified at the *int*-2 locus. Oncogene 4:1219–1224, 1989.
115. Tsujimoto Y, Yunis J, Onorato-Showe L, Erikson J, Nowell PC, Croce CM: Molecular

cloning of the chromosomal breakpoint of B cell lymphomas and leukemias with the t(11;14) chromosome translocation. Science 224:1403, 1984.
116. Tsujimoto Y, Jaffe E, Cossman J, Gorham J, Nowell PC, Croce RM: Clustering of breakpoints on chromosome 11 in human B cell neoplasms with the t(11;14) chromosomal translocation. Nature 315:340–343, 1985.
117. Ali IU, Merlo G, Lidereau R, Callahan R: The amplification unit on chromosome 11q13 in aggressive primary human breast tumors entails the *bcl*-1, *int*-2 and *hst* loci. Oncogene 4:89–92, 1989.
118. Theillet C, Adnane J, Szepetowski P, Simon M-P, Jeanteur P, Birnbaum D, Gaudray P: *Bcl*-1participates in the 11q13 amplification found in breast cancer. Oncogene 5:147–149, 1990.
119. Arnold A, Kim WG, Gaz RD, Eddy RL, Fukushima Y, Byers MG, Shows TB, Kronenberg HM: Molecular cloning and chromosomal mapping of DNA rearranged with the parathyroid hormone gene in a parathyroid adenoma. J Clin Invest 83:2034–2040, 1989.
120. Lammie GA, Fantl V, Smith R, Shuuring E, Brookes S, Michalides R, Dickson C, Arnold A, Peters G: D11S287, a putative oncogene on chromosome 11q13 is amplified and expressed in squamous cell and mammary carcinomas and linked to *bcl*-1. Oncogene 6:439–444, 1991.
121. Motokura T, Bloom T, Kim HG, Juppner H, Ruderman JV, Kronenberg HM, Arnold A: A novel cyclin encoded by a *bcl*-1-linked candidate oncogene. Nature 350:512–515, 1991.
122. Withers DA, Harvey RC, Faust JB, Melnyk OI, Carey K, Meeker T: Characterization of a candidate *bcl*-1 gene. Mol Cell Biol 11:4846–4853, 1991.
123. Matsushime H, Roussel MF, Ashmun RA, Sherr CJ: Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell 65:701–713, 1991.
124. Xiong Y, Connolly T, Futcher B, Beach D: Human D-type cyclin. Cell 65:691–699, 1991.
125. Lew DJ, Dulic V, Reed SI: Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. Cell 66:1197–1206, 1991.
126. Faust JB, Meeker TC: Amplification and expression of the *bcl*-1 gene in human solid tumor cell lines. Cancer Res 52:2460–2463, 1992.
127. Richard CW, Withers DA, Meeker TC, Maurer S, Evans G, Myers RM, Cox DR: A radiation hybrid map of the proximal long arm of human chromosome 11 containing the MEN-1 and *bcl*-1 disease loci. Am J Human Genet 49:1189–1196, 1991.
128. Moscow JA, Townsend AJ, Goldsmith ME, Whang-Peng J, Vickers PJ, Poisson R, Legault-Poison S, Myers CE, Cowan KH: Isolation of the human anionic glutathione S-transferase cDNA and the relation of its gene expression to estrogen receptor content in primary breast cancer. Proc Natl Acad Sci USA 85:6518–6522, 1988.
129. Hill DL: *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU), a substrate for glutathione (GSH) *S*-transferase. Proc Am Assoc Cancer Res 17:52, 1976.
130. Dulik DM, Fenselau C, Hilton J: Characterization of melphalan-glutathione adducts whose formation is catalyzed by glutathione transferases. Biochem Pharmacol 35: 3405–3409, 1986.
131. Shea TC, Kelley SC, Henner WD: Identification of an anionic form of glutathione transferase present in many human tumors and tumor cell lines. Cancer Res 48:527–533, 1988.
132. Dillio C, Del Boccio G, Aceto A, Cassacia R, Mucilli F: Elevation of glutathione transferase activity in human lung tumor. Carcinogenesis 9:335–340, 1988.
133. Moscow JA, Fairchild CR, Madden MJ, Ramson DT, Wieand HS, O'Brien EE, Poplack DG, Cossman J, Myers CE, Cowan KH: Expression of anionic glutathione S-transferase and P-glycoprotein genes in human tissues and tumors. Cancer Res 49:1422–1428, 1989.
134. Toffoli G, Frustaci S, Tumiotto L, Talamini R, Gherlinzoni F, Picci P, Boiocchi M: Expression of MDR1 and GST-π in human soft tissue sarcomas: Relation to drug resistance and biologic aggressiveness. Ann Oncol 3:63–69, 1992.
135. Saint-Ruf C, Malfoy B, Scholl S, Zafrani B, Dutrillaux B: GST-π gene is frequently

coamplified with *INT*-2 and *HST*F1 proto-oncogenes in human breast cancers. Oncogene 6:403–406, 1991.
136. Taub R, Kirsch I, Morton C, Lenoir G, Swan D, Tronick S, Aaronson S, Leder P: Translocation of the c-*myc* gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci USA 79: 7837–7841, 1982.
137. Dalla-Favera R, Bregni M, Erikson J, Patterson D, Gallo RC, Croce CM: Human c-*myc* *onc* gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci USA 79:7824–7827, 1982.
138. Dalla-Favera R, Wong-Staal F, Gallo R: Myc gene amplification in promyelocytic leukaemic cell line HL-60 and primary leukaemic cells of the same patient. Nature 299:61–63, 1982.
139. Collins S, Grondine M: Amplification of endogenous *myc*-related DNA sequences in a human myeloid leukaemia cell line. Nature 298:679–681, 1982.
140. Little CD, Nau MM, Carney DN, Gazdar AF, Minna JD: Amplification and expression of the c-*myc* oncogene in human lung cancer cell lines. Nature 306:194–196, 1983.
141. Alitalo K, Schwab M, Lin CC, Varmus HE, Bishop JM: Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-*myc*) in malignant neuroendocrine cells from a human colon carcinoma. Proc Natl Acad Sci USA 80:1707–1711, 1983.
142. Slamon DJ, deKernion JB, Verna IM, Cline MJ: Expression of cellular oncogenes in human malignancies. Science 224:256–262, 1984.
143. Kelly K, Cochran BH, Stiles CD, Leder P: Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35:603–610, 1983.
144. Lacy J, Sarkar SN, Summers WC: Induction of c-*myc* expression in human B lymphocytes by B-cell growth factor and anti-immunoglobulin. Proc Natl Acad Sci USA 83:1458–1462, 1986.
145. Concience J-F, Verrier B, Martin G: Interleukin-3-dependent expression of the c-*myc* and c-*fos* proto-oncogenes in hematopoietic cell lines. EMBO J 5:317–323, 1986.
146. Makino R, Hayashi K, Sugimura T: c-*myc* transcript is induced in rat liver at a very early stage of regeneration or by cycloheximide treatment. Nature 312:716–720, 1984.
147. Stewart TA, Pattengale PK, Leder P: Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MMTV/*myc* fusion genes. Cell 38:627–637, 1984.
148. Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P: Coexpression of MMTV/v-Ha-*ras* and MMTV/c-*myc* in transgenic mice: Synergistic actions of oncogenes in vivo. Cell 49:465–475, 1987.
149. Schonenberger CA, Andres AC, Groner B, Van der Valk M, Le Meur M, Gerlinger P: Targeted c-*myc* gene expression in mammary glands of transgenic mice induces mammary tumors with constitutive milk protein gene transcription. EMBO J 7:169–175, 1988.
150. Liu E, Santos G, Lee WMF, Osborne CK, Benz CC: Effects of c-*myc* overexpression on the growth characteristics of MCF-7 human breast cancer cells. Oncogene 4:979–984, 1989.
151. Santos GF, Scott GK, Lee WMF, Liu E, Benz CC: Estrogen-induced post transcriptional modulation of c-*myc* proto-oncogene expression in human breast cancer cells. J Biol Chem 263:9565–9568, 1988.
152. Van der Berg B, Van Selm-Miltenberg AJ, DeLaat SW, Van Zoelen EJ: Direct effects of estrogen on c-*fos* and c-*myc* protooncogene expression and cellular proliferation in human breast cancer cells. Mol Cell Endocrinol 64:223–228, 1988.
153. Wong MS, Murphy LC: Differential regulation of c-*myc* by progestins and antiestrogens in T-470 human breast cancer cells. J Steroid Biochem Mol Bio 39:39–44, 1991.
154. Watson PH, Pon RT, Shiu RPC: Inhibition of c-*myc* expression by phosphorothioate antisense oligonucleotide identifies a critical role for c-*myc* in the growth of human breast cancer. Cancer Res 51:3996–4000, 1991.
155. Benz CC, Scott GK, Santos GF, Smith HS: Expression of c-*myc*, c-Ha-*ras* and c-*erb*B-2

proto-oncogenes in normal and malignant breast epithelial cells. J Natl Cancer Inst 81:1704–1709, 1989.
156. Kozbor D, Croce CM: Amplification of the c-*myc* oncogene in one of five human breast carcinoma cell lines. Cancer Res 44:438–441, 1984.
157. Modjtahedi N, Lavialle C, Poupon M-F, Landin R-M, Cassingena R, Monier R, Brisson O: Increased level of amplification of the c-*myc* oncogene in tumors induced in nude mice by a human breast carcinoma cell line. Cancer Res 45:4372–4379, 1985.
158. Escot C, Theillet C, Lidereau R, Spyratos F, Champeme M-H, Gest J, Callahan R: Genetic alteration of the c-*myc* protooncogene (*myc*) in human primary breast carcinomas. Proc Natl Acad Sci USA 83:4834–4838, 1986.
159. Zhou D-J, Carre MJ: Proto-oncogene abnormalities in human breast cancer: c-*erb*B-2 amplification does not correlate with recurrence of disease. Oncogene 4:105–108, 1989.
160. Bonilla M, Ramirez M, Lopez-Cueto J, Gariglio P: In vivo amplification and rearrangement of c-*myc* oncogene in human breast tumors. J Natl Cancer Inst 80:665–671, 1988.
161. Cline MJ, Battifora H, Yokota J: Proto-oncogene abnormalities in human breast cancer: Correlations with anatomic features and clinical course of disease. J Clin Oncol 5: 999–1006, 1987.
162. Varley JM, Swallow JE, Brammar WJ, Whittaker JL, Walker RA: Alterations to either c-*erb*B-2 (*neu* or c-*myc* proto-oncogenes in breast carcinomas correlate with poor short-term prognosis. Oncogene 1:423–430, 1987.
163. Tsuda H, Hirohashi S, Hirota T, Shimosato Y: Alterations in copy number of c-*erb*B-2 and c-*myc* proto-oncogenes in advanced stage of human breast cancer. Acta Pathol Japon 41:19–23, 1991.
164. Donovan-Peluso M, Contento AM, Tobon H, Ripepi B, Locker J: Oncogene amplification in breast cancer. Am J Pathol 138:835–45, 1991.
165. Garcia I, Dietrich PY, AApro M, Vauthier G, Vadas L, Engel E: Genetic alterations of c-*myc*, c-*erb*B-2 and c-Ha-*ras* proto-oncogenes and clinical associations in human breast carcinomas. Cancer Res 49:6675–6679, 1989.
166. Tavassoli M, Quirke P, Farzaneh F, Lock NJ, Mayne LV, Kirkham N: c-*erb*B-2/c-*erb*A co-amplification indicative of high tumor grade in human breast carcinoma. Br J Cancer 60:505–510, 1989.
167. Brouillet JP, Theillet C, Maudelonde T, Defrenne A, Simony-Lafontaine J, Sertour J, Pujol H, Jeanteur P, Rochefort H: Cathepsin D assay in primary breast cancer and lymph nodes: Relationship with c-*myc*, c-*erb*B-2 and *int*-2 oncogene amplification and node invasiveness. Eur J Cancer 26:437–441, 1990.
168. Guerin M, Barrois M, Terrier MJ, Spielman M, Riou G: Overexpression of either c-*myc* or c-*erb*B-2/*neu* proto-oncogenes in human breast carcinomas: A correlation with poor prognosis. Oncol Res 3:21–31, 1988.
169. LeRoy X, Escot C, Brouillet JP, Theillet C, Maudelonde T, Simony-Lafontaine J, Pujol H, Rochefort H: Decrease of c-*erb*B-2 and c-*myc* RNA levels in tamoxifen-treated breast cancer. Oncogene 6:431–437, 1991.
170. Spandidos DA, Yiagnisis M, Papadimitriou K, Field JK: *ras*, c-*myc* and c-*erb*B-2 oncoproteins in human breast cancer. Anticancer Res 9:1385–1893, 1989.
171. Locker AP, Dowle CS, Ellis IO, Elston CW, Blamey RW, Sikara K, Evan G, Robins RA: c-*myc* oncogene product expression and prognosis in operable breast cancer. Br J Cancer 60:669–672, 1989.
172. Spandidos DA, Pintzas A, Kakkanas A, Yiagnisis M, Mahera E, Patra E, Agnantis NJ: Elevated expression of the myc gene in human benign and malignant lesions compared to normal tissue. Anticancer Res 7:1299–1304, 1987.
173. Papamichalis G, Francia K, Karachaliou FF, Anastassaides OTL, Spandidos DA: Expression of the c-*myc* oncoprotein in human metaplastic epithelial cells of fibrocystic disease. Anticancer Res 8:1223–1228, 1989.
174. Spandidos DA, Field JK, Agnantis NF, Evan GI, Moore JP: High levels of c-*myc* oncoprotein in human breast tumors determined by a sensitive ELISA technique.

Anticancer Res 9:821–826, 1989.
175. Bos JL: *Ras* oncogenes in human cancer: A review. Cancer Res 49:4682–4689, 1989.
176. Bos JL, Fearon ER, Hamilton SR, Verlaan-de Vries M, van Boom JW, van der Eb AJ, Vogelstein B: Prevalence of *ras* gene mutations in human colorectal cancers. Nature 327:293–297, 1987.
177. Forrester K, Almoguera C, Han K, Grizzle WE, Perucho M: Detection of high incidence of K-*ras* oncogenes during human colon tumorigenesis. Nature 327:298–303, 1987.
178. Almoguera C, Shibata D, Forrester K, Martin J, Annheim N, Perrucho M: Most human carcinomas of the exocrine pancreas contain mutant c-K-*ras* genes. Cell 53:549–554, 1988.
179. Rodenhuis S, Van de Wetering ML, Mooi WJ, Evers SG, Van Zandwijk N, Bos JL: Mutational activation of the K-*ras* oncogene: A possible pathogenetic factor in adenocarcinoma of the lung. N Engl J Med 317:929–935, 1987.
180. Rochlitz CF, Scott GK, Dodson JM, Liu E, Dollbaum C, Smith HS, Benz CC: Incidence of activating *ras* oncogene mutations associated with primary and metastatic human breast cancer. Cancer Res 49:357–360, 1989.
181. Sukumar S, Notario V, Martin-Zanka D, Barbacid M: Induction of mammary carcinomas in rats by nitroso-methyl urea involves malignant activation of H-*ras*-1 locus by single point mutations. Nature 306:658–661, 1983.
182. Zarble H, Sukumar S, Arthur AV, Martin-Zanca D, Barbacid M: Direct mutagenesis of Ha-*ras*-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. Nature 315:382–385, 1988.
183. Andres AC, Schoenenberger CA, Groner B, Hennighausen L, LeMeur M, Gerlinger P: Ha-*ras* oncogene expression directed by a milk protein promoter: Tissue specificity, hormonal regulation and tumor induction in transgenic mice. Proc Natl Acad Sci USA 84:1299–1303, 1987.
184. Andres AC, Behini O, Schubaur B, Dolder B, LeMeur M, Gerlenger P: H-*ras* induced transformation of mammary epithelium is favoured by increased oncogene expression or by inhibition of mammary regression. Oncogene 6:771–779, 1991.
185. Kasid A, Lippman ME, Papageorge AG, Lowy DR, Gelmann EP: Transfection of v-*ras*-H DNA into MCF-7 human breast cancer cells bypasses dependence on estrogen for tumorigenicity. Science 228:725–728, 1985.
186. Isonishi S, Hom DK, Thiebaut FB, Mann SC, Andrews PA, Basu A, Lazo JS, Eastman A, Howell SB: Expression of the c-Ha-*ras* oncogene in mouse NIH3T3 cells produces resistance to cisplatinum. Cancer Res 51:5903–5909, 1991.
187. Thor A, Ohuchi N, Horan Hand P, Callahan R, Weeks MO, Theillet C, Lidereau R, Escot C, Page D, Vilasi V, Schlom J: *ras* gene alterations and enhanced levels of ras p21 expression in a spectrum of benign and malignant human mammary tissues. Lab Invest 55:603–615, 1986.
188. Saglio G, Camaschella C, Giai M, Serra A, Guerrasio A, Peirone B, Gasparini P, Mazza U, Cappellini R, Biglia N: Distribution of Ha-*ras*-1 proto-oncogene alleles in breast cancer patients and a control population. Breast Cancer Res Treat 11:147–153, 1988.
189. Spandidos DA, Agnantis NJ: Human malignant tumors of the breast as compared to their normal tissue, have elevated expression of the Harvey-*ras* oncogene. Anticancer Res 4:269–272, 1984.
190. Biunno I, Pozzi MR, Pierotti MA, Pilotti S, Cattoretti G, Della Porta G: Structure and expression of oncogenes in surgical specimens of human breast carcinomas. Br J Cancer 57:464–468, 1988.
191. Whittaker JL, Walker RA, Varley JM: Differential expression of cellular oncogenes in benign and malignant human breast tissue. Int J Cancer 38:651–655, 1986.
192. Agnantis NJ, Parissi P, Anagnostakis D, Spandidos DA: Comparative study of Harvey *ras* oncogene expression with conventional clinicopathological parameters of breast cancer. Oncology 43:36–39, 1986.
193. Horan Hand P, Vilasi V, Thor A, Ohuchi N, Schlom J: Quantitation of Harvey *ras* p21 enhanced expression in human breast and colon carcinomas. J Natl Cancer Inst 79:59–65, 1987.

194. Agnantis NJ, Petraki C, Markoulatos P, Spandidos DA: Immunohistochemical study of the *ras* oncogene expression in human breast lesions. Anticancer Res 6:1157–1160, 1986.
195. Ohuchi N, Thor A, Page DI, Horan Hand P, Halter S, Schlom J: Expression of the 21,000 molecular weight *ras* protein in a spectrum of benign and malignant human mammary tissues. Cancer Res 46:2511–2519, 1986.
196. Clair T, Miller WR, Cho-Chung YS: Prognostic significance of the expression of a *ras* protein with a molecular weight of 21,000 by human breast cancer. Cancer Res 47:5290–5293, 1987.
197. Lundy J, Grimson R, Mishriki Y, Chao S, Oravez S, Fromowitz F, Viola MV: Elevated *ras* oncogene expression correlates with lymph node metastases in breast cancer patients. J Clin Oncol 4:1321–1325, 1986.
198. Watson DM, Elton RA, Jack WJ, Dixon JM, Chetty U, Miller WR: The H-*ras* oncogene product p21 and prognosis in human breast cancer. Breast Cancer Res Treat 17:161–169, 1991.
199. Dati C, Muraca R, Tazartes OM, Antoniotti S, Perrofeau I, Giai M, Cortese P, Sismondi Saglio S, Di Bartoli M: c-*erb*B-2 and *ras* expression levels in breast cancer are correlated and show a co-operative association with unfavorable clinical outcome. Int J Cancer 47:833–838, 1991.
200. Hainsworth PJ, Henderson MA, Stillwell RG, Bennet RC: Comparison of EGFR, c-*erb*B-2 product and *ras* p21 immunohistochemistry as prognostic markers in primary breast cancer. Eur J Surg Oncol 17:9–15, 1991.
201. Querzoli P, Marchetti E, Bagni A, Marzola A, Fabris G, Nanci I: Expression of p21 *ras* gene products in breast cancer relates to histological types and to receptor and nodal status. Breast Cancer Res Treat 12:23–30, 1988.
202. Tanaka T, Slamon DJ, Battifora H, Cline MJ: Expression of p21 *ras* oncoprotein in human cancers. Cancer Res 46:1465–1470, 1986.
203. Candlish W, Kerr IB, Simpson HW: Immunocytochemical demonstration and significance of p21 *ras* family oncogene product in benign and malignant breast disease. J Pathol 150:163–167, 1986.
204. Horan Hand P, Thor A, Wienderlich D, Muravo R, Caruso A, Schlom J: Monoclonal antibodies of predefined specificity detects activated *ras* gene expression in human mammary and colon carcinoma. Proc Natl Acad Sci USA 81:5227–5231, 1984.
205. Fromowitz FB, Viola MV, Chao S, Oravez S, Mishriki Y, Finkel G, Grimson R, Lundy J: *ras* p21 expression in the progression of breast cancer. Human Pathol 18:1268–1275, 1987.
206. Czerniak B, Chen R, Tuziak K, Markrewski M, Kram A, Gorczyca W, Deitch C, Herz F, Koss LG: Expression of *ras* oncogene p21 protein in relation to regional spread of human breast carcinomas. Cancer 63:2008–2013, 1989.
207. Nobumoto W, Vande Woude GF, Ikawa Y, Sagata N: Specific proteolysis of the c-*mos* proto-oncogene product by calpain on fertilization of xenopus eggs. Nature 342:505–511, 1989.
208. Sagata N, Watanabe N, Vande Woude GF, Ikawa Y: The c-*mos* proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. Nature 342:512–518, 1989.
209. Lidereau R, Mathieu-Mahul D, Theillet C, Renaud M, Mauchauffe M, Gest J, Larsen CJ: Presence of an allelic Eco RI restriction fragment of the c-*mos* locus in leukocyte and tumor cells DNAs of breast cancer patients. Proc Natl Acad Sci USA 82:7068–7070, 1985.
210. Lidereau R, Cole ST, Larsen CJ, Mathieu-Mahul D: A single point mutation responsible for c-*mos* polymorphism in cancer patients. Oncogene 1:235–237, 1987.
211. Hall JM, Zuppan PJ, Anderson LA, Huey B, Carter C, King M-C: Oncogenes and human breast cancer. Am J Hum Genet 44:577–584, 1989.
212. Curran T, Franza BR: *Fos* and *jun*: The AP-I connection. Cell 55:395–397, 1988.
213. Sorokine I, Ben-Mahrez K, Bracone A, Thierry D, Ishii S, Imamoto F, Kohiyama M: Presence of circulating anti-c-*myb* oncogene product antibodies in human sera. Int J Cancer 47:665–669, 1991.
214. Guerin M, Sheng Z-M, Andrieu N, Riou G: Strong association between c-*myb* and

estrogen receptor expression in human breast cancer. Oncogene 5:131–135, 1990.
215. Adnane J, Gaudray P, Dionne CA, Crumley G, Jaye M, Schlessinger J, Jeanteur P, Birnbaum D, Theillet C: BEK and FLG, two receptors to members of the FGF family, are amplified in subsets of human breast cancers. Oncogene 6:659–666, 1991.
216. Shih C, Padhy LC, Murray M, Weinberg RA: Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. Nature 290:261–264, 1981.
217. Schecter AC, Stern BF, Vaidyanathan L, Decker SJ, Drebin JA, Greene MI, Weinberg RA: The *neu* oncogene: An *erb*-B-related gene encoding a 185,000-M_r tumour antigen. Nature 312:513–516, 1984.
218. Schecter, Hung M-C, Vaidyanathan L, Weinberg RA, Yang-Feng TL, Francke U, Ullrich A, Coussens L: The *neu* gene: An *erb*B-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. Science 976–978, 1985.
219. Coussens L, Yang-Feng TL, Liao Y-C, Chen E, Gray A, McGrath J, Seeberg PH, Libermann TW, Schlessinger J, Francke U, Levinson A, Ullrich A: Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science 230:1132–1139, 1985.
220. Yamamoto T, Ikawa S, Akiyama T, Semba K, Nomura N, Miyajima N, Saito T, Toyoshima K: Similarity to protein encoded by the human c-*erb*B-2 gene to epidermal growth factor receptor. Nature 319:230–234, 1986.
221. DiFiore PP, Pierce JW, Kraus MH, Segatto O, King CR, Aaronson SA: *Erb*B-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science 237:178–182, 1987.
222. Stern DF, Kamps MP, Cao H: Oncogenic activation of p185 *neu* stimulates tyrosine phosphorylation in vivo. Mol Cell Biol 5:3969–3973, 1988.
223. Wildenhain Y, Pawson T, Blackstein ME, Andrulis IL: p185 *neu* is phosphorylated on tyrosine in human breast tumors which overexpress *neu*/*erb*B-2. Oncogene 879–883, 1990.
224. Sarup JC, Johnson RM, King KL, Fendly BM, Lipari MT, Napier MA, Ullrich A, Shepard HM: Characterization of an anti-$p185^{HER2}$ monoclonal antibody that stimulates receptor function and inhibits tumor cell growth. Growth Reg 1:72–82, 1991.
225. Scott GK, Dodson JM, Montgomery PA, Johnson RM, Sarup JC, Wong WL, Ullrich A, Shepard HM, Benz CC: $p185^{HER2}$ signal transduction in breast cancer cells. J Biol Chem 266:14300–14305, 1991.
226. Bargmann CI, Hung M-C, Weinberg RA: Multiple independent activations of the *neu* oncogene by a point mutation altering the transmembrane domains of p185. Cell 73: 649–657, 1986.
227. Bargmann CI, Weinberg RA: Oncogenic activation of the *neu*-encoded receptor protein by point mutation and deletion. EMBO J 7:2043–2052, 1988.
228. Weiner DB, Liu J, Cohen JA, Williams WV, Greene MI: A point mutation in the *neu* oncogene mimics ligand induction of receptor aggregation. Nature 339:230–231, 1989.
229. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P: Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-*neu* oncogene. Cell 54:105–115, 1988.
230. Bouchard L, Lamarre L, Tremblay PJ, Jolicoeur P: Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-*neu* oncogene. Cell 57:931–936, 1989.
231. King CR, Kraus MH, Aaronson SA: Amplification of a novel v-*erb*B-related gene in a human mammary carcinoma. Science 229:974–976, 1985.
232. Fukushige S-I, Matsubara K-I, Yoshida M, Sasaki M, Suzuki T, Semba K, Toyoshima K, Yamamoto T: Localization of a novel v-*erb*B-related gene, c-*erb*B-2, on human chromosome 17 and its amplification in a gastric cancer cell line. Mol Cell Biol 6:955–958, 1986.
233. Semba K, Kamata N, Toyoshima K, Yamamoto T: A v-*erb*B-related proto-oncogene c-*erb*B-2, is distinct from the c-*erb*B-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc Natl Acad Sci USA 82:6497–6501, 1985.
234. Kraus MH, Popescu NC, Amsbaugh SC, King CR: Overexpression of the EGF receptor-

related proto-oncogene *erb*B-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J 6:605–610, 1987.
235. Tal M, King CR, Kraus MH, Ullrich A, Schlessinger J, Givol D: Human HER2 (*neu*) promoter: Evidence for multiple mechanisms for transcriptional initiation. Mol Cell Biol 7:2597–2601, 1987.
236. Kallioniemi O-P, Kallioniemi A, Chen L-C, Thor A, Kurisu W, Thor A, Chen LC, Smith HS, Waldman FM, Pinkel D, Gray JW: ERBB2 amplification in breast cancer analyzed by fluorescence in situ hybridization. Proc Natl Acad Sci USA 89:5321–5325, 1992.
237. Lemoin NR, Staddon S, Dickson S, Barnes DM, Gullick WJ: Absence of activating transmembrane mutations in the c-*erb*B-2 proto-oncogene in human breast cancer. Oncogene 5:237–239, 1990.
238. Saya H, Ara S, See PS, Ro J, Hung M-C: Direct sequencing analysis of transmembrane regions of the human *neu* gene by polymerase chain reaction. Mol Carcinogen 3:198–201, 1990.
239. Hudziak RM, Schlessinger J, Ullrich A: Increased expression of the putative growth factor receptor $p185^{HER2}$ causes transformation and tumorigenesis of NIH3T3 cells. Proc Natl Acad Sci USA 84:7159–7163, 1987.
240. Hudziak RM, Lewis GD, Shalaby MR, Eessalu TE, Aggarwal BB, Ullrich A, Shepard HM: Amplified expression of the HER2/*erb*B-2 oncogene induces resistance to tumor necrosis factor α in NIH3T3 cells. Proc Natl Acad Sci USA 85:5102–5106, 1988.
241. Segatto O, King CR, Pierce JH, DiFiore PP, Aaronson SA: Different structural alterations upregulate in vitro tyrosine kinase activity and transforming potency of the *erb*B-2 gene. Mol Cell Biol 8:5570–5574, 1988.
242. Wildenhain Y, Pawson T, Blackstein ME, Andrulis IL: p185*neu* is phosphorylated on tyrosine in human primary breast tumors which overexpress neu/*erb*B-2. Oncogene 5: 879–883, 1990.
243. Suda Y, Aizawa S, Furuta Y, Yagi T, Ikawa Y, Saitoh K, Yamada Y, Toyoshima K, Yamamoto T: Induction of a variety of tumors by c-*erb*B-2 and clonal nature of lymphomas even with the mutated gene (Val^{659}-Glu^{659}). EMBO J 9:181–190, 1990.
244. Pierce JH, Arnstein P, DiMarco E, Artrip J, Kraus MH, Lenardo F, DiFiore PP, Aaronson SA: Oncogenic potential of *erb*B-2 in human mammary epithelial cells. Oncogene 6:1189–1194, 1991.
245. Benz CC, Scott GK, Sarup JCK, Johnson RM, Tripathy D, Coronado E, Shepard HM, Osborne CK: Estrogen-dependent tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/*mev*. Breast Cancer Res. Treatment. In press, 1992.
246. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235:177–182, 1987.
247. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF: Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. Science 244:707–712, 1989.
248. Berchuck A, Kamel A, Whitaker R, Kerns B, Olt G, Kinney R, Soper JT, Dodge R, Clarke-Pearson DL, Marks P, McKenzie S, Yin S, Bast RC: Overexpression of HER-2/*neu* associated with poor survival in advanced epithelial ovarian cancer. Cancer Res 50:4087–4091, 1990.
249. Kern JA, Schwartz DA, Nordberg JE, Weiner DB, Greene MI, Torney L, Robinson RA: p185*neu* expression in human lung adenocarcinomas predicts shortened survival. Cancer Res 50:5184–5191, 1990.
250. Borg A, Tandon AK, Sigurdsson H, Clark GM, Ferno Marten, Fuqua SAW, Killander D, McGuire WL: HER-2/*neu* amplification predicts poor survival in node-positive breast cancer. Cancer Res 50:7332–7337, 1990.
251. Tandon AK, Clark GM, Chamness GC, Ullrich A, McGuire WL: HER-2/*neu* oncogene protein and prognosis in breast cancer. J Clin Oncol 7:1120–1128, 1989.
252. Thor AD, Schwartz LH, Koerner FC, Edgerton SM, Skates SJ, Yin S, McKenzie SJ,

Panicali DL, Marks PJ, Fingert HF, Wood WC: Analysis of c-*erb*B-2 expression in breast carcinomas with clinical follow-up. Cancer Res 49:7147–7152, 1989.
253. Wright C, Angus B, Nicholson S, Sainsbury JRC, Cairns J, Gullick WJ, Kelley P, Harris AH, Horne CHW: Expression of c-*erb*B-2 oncoprotein: A prognostic indicator in human breast cancer. Cancer Res 49, 2087–2090, 1989.
254. Gullick WJ, Love SB, Wright C, Barnes DM, Gusterson B, Harris AL, Altman DG: c-*erb*B-2 protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. Br J Cancer 63:434–438, 1991.
255. Anbazhagan R, Gelber RD, Bettelheim R, Goldhirsch A, Gusterson BA: Association of c-*erb*B-2 expression and S-phase fraction in the prognosis of node positive breast cancer. Ann Oncol 2:47–53, 1991.
256. Walker RA, Gullick WJ, Varley JM: An evaluation of immunoreactivity for c-*erb*B-2 protein as a marker of poor short-term prognosis in breast cancer. Br J Cancer 60: 426–429, 1989.
257. Borresen A-L, Ottestad L, Gaustad A, Andersen TI, Heikkila R, Jahnsen T, Tveit KM, Nesland JM: Amplification and protein over-expression of the *neu*/HER-2/c-*erb*B-2 protooncogene in human breast carcinomas: Relationship to loss of gene sequences on chromosome 17, family history and prognosis. Br J Cancer 62:585–590, 1990.
258. McCann AH, Dervan PA, O'Regan M, Codd MB, Gullick WJ, Tobin BM, Carney DN: Prognostic significance of c-*erb*B-2 and estrogen receptor status in human breast cancer. Cancer Res 51:3296–3303, 1991.
259. Dykins R, Corbett IP, Henry JA, Wright C, Yuan J, Henessy C, Lennard TJ, Angus B, Horne CH: Long-term survival in breast cancer related to overexpression of the c-*erb*B-2 oncoprotein: An immunohistochemical study using monoclonal antibody NCL-CBII. J Pathol 163:105–110, 1991.
260. Paik S, Hazan R, Fisher ER, Sass RE, Fisher B, Redmond C, Schlessinger J, Lippman ME, King CR: Pathologic findings from the National Surgical Adjuvant Breast and Bowel Project: Prognostic significance of *erb*B-2 protein overexpression in primary breast cancer. J Clin Oncol 8:103–112, 1990.
261. Ro J, El-Naggar A, Ro JY, Blick M, Frye D, Fraschini G, Fritsche H, Hortobagyi G: c-*erb*B-2 amplification in node-negative human breast cancer. Cancer Res 49:6941–6944, 1989.
262. Parks HC, Lillycrop K, Howell A, Craig RK: c-*erb*B-2 mRNA expression in human breast tumors: Comparison with c-*erb*B-2 DNA amplification and correlation with prognosis. Br J Cancer 61:39–45, 1990.
263. Lovekin C, Ellis IO, Locker A, Robertson JFR, Bell J, Nicholson R, Gullick WJ, Elston CW, Blamey RW: c-*erb*B-2 oncoprotein expression in primary and advanced breast cancer. Br J Cancer 63:439–443, 1991.
264. Wistanley J, Cooke T, Murray GD, Platt-Higgins A, George WD, Holt S, Myskov M, Spedding A, Barraclough Br, Rudland PS: The long term prognostic significance of c-*erb*B-2 in primary breast cancer. Br J Cancer 63:447–450, 1991.
265. Rilke F, Colnaghi MI, Cascinelli N, Andreola S, Baldini MT, Bufalino R, Della Porta G, Menard S, Pierotti MA, Testori A: Prognostic significance of HER-2/*neu* expression in breast cancer and its relationship to other prognostic factors. Int J Cancer 49:44–49, 1991.
266. O'Reilly SM, Barnes OM, Camplejohn RS, Bartkova J, Gregory WM, Richards MA: The relationship between c-*erb*B-2 expression, S-phase fraction and prognosis in breast cancer. Br J Cancer 63:444–446, 1991.
267. May E, Mouriesse H, May-Levin F, Qian JF, May P, Delarve JC: Human breast cancer; identification of populations with a high risk of early relapse in relation to both oestrogen receptor status and c-*erb*B-2 overexpression. Br J Cancer 62:430–435, 1990.
268. Ali IU, Campbell G, Lidereau R, Callahan R: Lack of evidence for the prognostic significance of c-*erb*B-2 amplification in human breast carcinoma. Oncogene Res 3:139–146, 1988.
269. Heintz NH, Leslie KO, Rogers LA, Howard PL: Amplification of the c-*erb*B-2 oncogene and prognosis of breast adenocarcinoma. Arch Pathol Lab Med 114:160–163, 1990.

270. Gusterson BA, Machin LG, Gullick WJ, Gibbs NM, Powles TJ, Elliot C, Ashley S, Monaghan P, Harrison S: c-*erb*B-2 expression in benign and malignant breast disease. Br J Cancer 58:453–457, 1988.
271. Van de Vijver MJ, Peterse JL, Mooi WJ, Wisman P, Lomans J, Dalesio O, Nusse R: *Neu*-protein overexpression in breast cancer. Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. N Engl J Med 391:1239–1245, 1988.
272. Barnes DM, Lammie GA, Millis RR, Gullick WJ, Allen DS, Altman DG: An immunohistochemical evaluation of c-*erb*B-2 expression in human breast carcinoma. Br J Cancer 58:448–452, 1988.
273. Paterson MC, Dietrich KD, Danyluk J, Paterson AGH, Lees AW, Jamil N, Hanson J, Jenkins H, Krause BE, McBlain WA, Slamon DJ, Fourney RM: Correlation between c-*erb*B-2 amplification and risk of recurrent disease in node-negative breast cancer. Cancer Res 51:556–567, 1991.
274. Richner J, Gerber HA, Locher GW, Goldhirsh A, Gelber RD, Gullick WJ, Berger MS, Groner B, Hynes NE: c-*erb*B-2 protein expression in node negative cancer. Annals Oncol 1:263–268, 1990.
275. Wright C, Nicholson S, Angus B, Sainsbury JRC, Farndon J, Cairns J, Harris AL, Horne CHW: Relationship between c-erbB2 protein product expression and response to endocrine therapy in advanced breast cancer. Br J Cancer 65:118–121, 1992.
276. Berns PMJJ, Klijn JGM, Van Staveren IL, Portengen H, Noordegraaf E, Foekens JA: Frequency and relevance of (onco)-gene amplification in human breast tumors. Proc Am Assoc Cancer Res 32:291, 1991.
277. Berger MS, Locker GW, Saurer S, Gullick WJ, Waterfield MD, Gronerl B, Hynes NE: Correlation of c-*erb*B-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. Cancer Res 48:1238–1243, 1989.
278. Guerin M, Gabillot M, Mathieu M-C, Travagli J-P, Spielmann M, Andrieu N, Riou G: Structure and expression of c-*erb*B-2 and EGF receptor genes in inflammatory and non-inflammatory breast cancer: Prognostic significance. Int J Cancer 43:201–208, 1989.
279. Querzoli P, Marchetti E, Fabris G, Marzola A, Ferretti S, Iacobelli S, Hazan R, King CR, Nenci I: Immunohistochemical expression of c-*erb*B-2 in human breast cancer by monoclonal antibody: Correlation with lymph node and ER status. Tumori 76:461–464, 1990.
280. Seshadri R, Matthews C, Dubrovic A, Horsfall DJ: The significance of oncogene amplification in primary breast cancer. Int J Cancer 43:270–272, 1989.
281. Lacroix H, Iglehart JD, Skinner MA, Kraus MH: Overexpression of *erb*B-2 or EGF receptor proteins in early stage mammary carcinoma is detected simultaneously in matched primary tumors and regional metastases. Oncogene 4:145–151, 1989.
282. Zeillinger R, Kury F, Czerwenka K, Kubista E, Sliutz G, Knogler W, Huber W, Zielinski C, Reiner G, Jakesz R, Staffen A, Reiner A, Wrba F, Spona F: HER-2 amplification steroid receptors and epidermal growth factor receptor in primary breast cancer. Oncogene 4:109–114, 1989.
283. Rio MC, Bellocq JP, Gairard B, Rasmussin UB, Krust A, Koehl C, Calderoli H, Schiff V, Renaud R, Chambon P: Specific expression of the pS2 gene in subclasses of breast cancers in comparison with expression of the oestrogen and progesterone receptors and the oncogene *ERB*B-2. Proc Natl Acad Sci USA 84:9243–9247, 1987.
284. Borg A, Sigurasson H, Tandon AK, Clarke GM, Ferno M, Killander D, McGuire WL: Proto-oncogene amplification in human breast cancer. Nordic Cancer Union Symposium, Stockholm 1989 (abstr).
285. Bacus SS, Bacus JW, Slamon DJ, Press MF: HER-2/*neu* oncogene expression and DNA polidy analysis in breast cancer. Arch Pathol Lab Med 114:164–169, 1990.
286. Van Diest PJ, Baak JP, Chin D, Theeuwes JW, Bacus SS: Quantitation of HER-2/*neu* oncoprotein overexpression in invasive breast cancer by image analysis: A study comparing fresh and paraffin-embedded material. Anal Cell Pathol 3:195–202, 1991.

287. King CR, Swain SM, Porter L, Steinberg SM, Lippman ME, Gelmann EP: Heterogeneous expression of c-*erb*B-2 messenger RNA in human breast cancer. Cancer Res 49:4183–4191, 1989.
288. Venter DJ, Tuzi NL, Kumar S, Gullick WJ: Overexpression of the c-*erb*B-2 oncoprotein in human breast carcinomas: Immunohistological assessment correlates with gene amplification. Lancet 2:69–72, 1987.
289. Iglehart JD, Kraus MH, Langton BC, Huper G, Kerns BJ, Marks JR: Increased *erb*B-2 gene copies and expression in multiple stages of breast cancer. Cancer Res 50:6701–6707, 1990.
290. Hanna W, Kahn HJ, Andrulis I, Pawson T: Distribution and patterns of staining of *neu* oncogene product in benign and malignant breast diseases. Modern Pathol 3:455–461, 1990.
291. Barnes DM, Meyer JS, Gonzalez JG, Gullick WJ, Millis RR: Relationship between c-*erb*B-2 immunoreactivity and thymidine labelling index in breast carcinoma in situ. Breast Cancer Res Treat 18:11–17, 1991.
292. Borg A, Linell F, Idvall I, Johansson S, Sigurdsson H, Ferno M, Killander D: HER-2/*neu* amplification and comedo type breast carcinoma. Lancet 1:1268–1269, 1989.
293. Porter PL, Garcia R, Moe R, Corwin DJ, Gown AM: c-*erb*B-2 oncogene protein in in situ and invasive lobular breast neoplasia. Cancer 68:331–334, 1991.
294. Soomro S, Shousha S, Taylor P, Shepard HM, Feldmann M: c-*erb*B-2 expression in different histologic types of invasive carcinoma. J Clin Path 44:211–214, 1991.
295. Liu E, Thor A, He M, Barcos M, Ljung B-M, Benz C: The HER2 (c-*erb*B-2) oncogenes is frequently amplified in in situ carcinomas of the breast. Oncogene 7:1027–1032, 1992.
296. Drebin JA, Link VC, Stern DF: Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. Cell 41:695–706, 1985.
297. Drebin JA, Link VC, Weinberg RA: Inhibition of tumor growth by a monoclonal antibody reactive with oncogene-encoded tumor antigen. Proc Natl Acad Sci USA 83:9129–9133, 1986.
298. Drebin JA, Link VC, Greene MI: Monoclonal antibodies reactive with distinct domains of the *neu* oncogene-encoded p185 molecule exerts synergistic anti-tumor effects. Oncogene 2:273–277, 1988.
299. Drebin JA, Link VC, Greene MI: Monoclonal antibodies specific for the *neu* oncogene product directly mediate anti-tumor effects in vivo. Oncogene 2:387–394, 1988.
300. Bernards R, Destree A, MacKenzie S: Effective tumor immunotherapy directed against on oncogene-encoded product using a vaccine virus vector. Proc Natl Acad Sci USA 84:6854–6858, 1987.
301. Fendly BM, Kotts C, Velterlein D, Lewis GD, Winget M, Carver ME, Watson SR, Sarup J, Saks S, Ullrich A: The extracellular domain of HER2/*neu* is a potential immunogen for active specific immunotherapy of breast cancer. J Biol Resp Modif 9:449–455, 1990.
302. Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, Ullrich A: $p185^{HER2}$ monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. Mol Cell Biol 9:1165–1172, 1989.
303. Fendly BM, Winget M, Hudziak RM, Lipari MT, Napier MA, Ullrich A: Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor of HER2/*neu* gene product. Cancer Res 50:1550–1558, 1990.
304. Hancock MC, Langton BC, Chan T, Toy P, Monahan JJ, Mischak RP, Shawver LK: A monoclonal antibody against the c-*erb*B-2 protein enhance the cytotoxicity of *cis*-diamminedichloroplatinum against human breast and ovarian tumor cell lines. Cancer Res 51:4575–4380, 1991.
305. Colomer R, Lupu R, Gelmann EP: *erb*B-2 antisense oligonucleotides inhibit the growth of the breast carcinoma cells with amplified *erb*B-2. Proc Am Assoc Cancer Res 31:308, 1990.
306. Kraus MH, Issing W, Miki T, Popescu NC, Aaronson SA: Isolation and characterization of ERBB-3, a third member of the ERBB1 epidermal growth factor receptor family:

Evidence for overexpression in a subset of human mammary tumors. Proc Natl Acad Sci USA 86:9193–9197, 1989.
307. Carpenter G: Receptors for epidermal growth factor and polypeptide mitogens. Ann Rev Biochem 56:881–914, 1987.
308. Fisher DA, Lakshmanan J: Metabolism and effects of epidermal growth factor and related factors in mammals. Endocrin Rev 11:418–442, 1990.
309. Goustin AS, Leof EB, Shipley GD, Moses HL: Growth factors and cancer. Cancer Res 46:1015–1029, 1986.
310. DiFiore PP, Pierce JH, Flemming TP, Hazan R, Ullrich A, King CR, Schlessinger J, Aaronson SA: Overexpression of the human EGF receptor confers and EGF-dependent transformed phenotype to NIH3T3 cells. Cell 51:1063–1070, 1988.
311. Perroteau I, Salomon D, DeBortoli M, Kidwell W, Hazarika P, Pardue R, Dedman J, Tam J: Immunological detection and quantitation of alpha transforming growth factors in human breast carcinoma cells. Breast Cancer Res Treat 7:201–210, 1986.
312. Travers MT, Barrett-Lee PJ, Berger U, Luqmani YA, Gazet J-C, Powles TJ, Coombes RC: Growth factor expression in normal, malignant and benign tissue. Br Med J 296:1621–1624, 1988.
313. Matsui Y, Halter SA, Holt JT, Hogan BLM, Coffey RJ: Development of mammary hyperplasia and neoplasia in MMTV-TGF alpha transgenic mice. Cell 61:1147–1155, 1990.
314. Sandgren EP, Luetteke NC, Palmiter RD, Brinster RL, Lee DC: Overexpression of TGFα in transgenic mice: Induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. Cell 61:1121–1135, 1990.
315. Jhappan C, Stahle C, Harkins RN, Fausto N, Smith GH, Merlino GT: TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell 61:1137–1146.
316. Kurachi H, Okamoto S, Oka T: Evidence for the involvement of the submandibular gland epidermal growth factor in mouse mammary tumorigenesis. Proc Natl Acad Sci USA 82:5940–5943, 1985.
317. Klijn JGM, Berns PM, Schmitz PI, Foekens JA: The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: A review on 5232 patients. Endocrine Rev, 13:3–17, 1992.
318. Marx D, Schauer A, Reiche C, May A, Ummenhofer L, Reles A, Rauschecker H, Sauer R, Schumacher M: c-*erb*B-2 expression in correlation to other biological parameters of breast cancer. J Cancer Res Clin Oncol 116:15–20, 1990.
319. Nicholson S, Wright C, Sainsbury JRC, Halcrow P, Kelly P, Angus B, Farndon JR, Harris AL: Epidermal growth factor receptor (EGFr) as a marker for poor prognosis in node-negative breast cancer patients: *neu* and tamoxifen failure. J Steroid Biochem Mol Biol 37:811–814, 1990.
320. Bacus SS, Ruby SG, Weinberg DS, Chin D, Ortiz R, Bacus JW: HER-2/*neu* oncogene expression and proliferation in breast cancers. Ann J Pathol 137:103–111, 1990.
321. Kokai Y, Myers JN, Wada T, Brown VI, LeVea CM, Davis JG, Dobashi K, Greene MI: Synergistic interaction of p185c-*neu* and the EGF receptor leads to transformation of rodent fibroblasts. Cell 58:287–292, 1989.
322. Gullick WJ: New developments in the molecular biology of breast cancer. Eur J Cancer 26:509–510, 1990.
323. Stern DF, Kamps MP: EGF-stimulated tyrosine phosphorylation of p185^{neu}: A potential model for receptor interactions. EMBO J 7:995–1001, 1988.
324. Wada T, Qian X, Greene MI: Intermolecular association of the p185^{neu} protein and EGF receptor modulates EGF receptor function. Cell 61:1339–1347, 1990.
325. Yee D, Cullen KJ, Paik S, Perdue JF, Hampton B, Schwartz A, Lippman ME, Rosen N: Insulin-like growth factor II mRNA expression in human breast cancer. Cancer Res 48:6691–6696, 1988.
326. Karey KP, Sirbasku DA: Differential responsiveness of human breast cancer cell lines

MCF-7 and T47D to growth factors and 17-β estradiol. Cancer Res 48:4083–4092, 1988.
327. Yee D, Paik S, Lebovie G, Marcus R, Favoni R, Cullen R, Lippman ME, Rosen N: Analysis of IGF-I gene expression in malignancy — evidence for a paracrine role in human breast cancer. Mol Endocrinol 3:509–517, 1989.
328. Ullrich A, Bell JR, Chen FY, Herrera R, Petrozelli M, Pull TJ, Gray A, Coussens L, Liao Y-C, Tsubokawa M, Mason A, Seeburg PH, Grunfeld C, Rosen OM, Ramachandran J: Human insulin receptor and its relation to the tyrosine kinase family of oncogenes. Nature 313:756–761, 1985.
329. Massague J, Czech MP: The subunit structures of two distinct receptors for insulin like growth factors I and II and their relationship to the insulin receptor. J Biol Chem 257:5038–5045, 1982.
330. Morgan DO, Edman JC, Standring DN, Fried VA, Smith MC, Roth RA, Rutter WJ: Insulin-like factor II receptor as a multifunctional binding protein. Nature 329:301–307, 1987.
331. Pekonen F, Partanen S, Makinen T, Rutanen E-M: Receptors for epidermal growth factor and insulin-like growth factor I and their relation to steroid receptors in human breast cancer. Cancer Res 48:1343–1347, 1988.
332. Cullen KJ, Yee D, Sly WS, Perdue J, Hampton B, Lippman ME, Rosen N: Insulin-like growth factor receptor expression and function in human breast cancer. Cancer Res 50:48–53, 1990.
333. Papa V, Pezzino V, Constantino A, Belfiore A, Giuffrida D, Frittatta L, Vannelli GB, Brand R, Goldfine ID, Vigneri R: Elevated insulin receptor content in human breast cancer. J Clin Invest 86:1503–1510, 1990.
334. Fradkin JE, Eastman RC, Lesniak MA, Roth J: Specificity spillover at the hormone receptor — exploring its role in human disease. N Engl J Med 320:640–645, 1989.
335. Flier JS, Usher P, Moses AC: Monoclonal antibody to the type I insulin-like growth factor (IGF-I) blocks receptor-mediated DNA synthesis: Clarification of the mitogenic mechanisms of IGF-I and insulin in human skin fibroblasts. Proc Natl Acad Sci USA 83:664–668, 1986.
336. Daly RJ, Harris WH, Wang DY, Darbre PD: Autocrine production of insulin-like growth factor II using an inducible expression system results in reduced estrogen sensitivity of MCF-7 human breast cancer cells. Cell Growth Differ 2:457–464, 1991.
337. Bonneterre J, Peyrat JP, Beuscart R, Demaille A: Prognostic significance of insulin-like growth factor I receptors in human breast cancer. Cancer Res 50:6931–6935, 1990.
338. Foekens JA, Portengen H, van Putten WLJ, Trapman AMAC, Reubi JC, Alexieva-Figusch J, Klijn JGM: Prognostic value of receptors for insulin-like growth factor I, somatostatin and epidermal growth factor in human breast cancer. Cancer Res 49:7002–7009, 1989.
339. Giorgino F, Belfiore A, Milazzo G, Constantino A, Maddux B, Whittaker J, Goldfine ID, Vigneri R: Overexpression of insulin receptors in fibroblasts and ovary cells induces a ligand-mediated transformed phenotype. Mol Endocrinol 5:452–459, 1991.
340. Hennipman A, van Oirschot BA, Smits J, Rijksen G, Staal GEJ: Tyrosine kinase activity in breast cancer, benign breast disease and normal breast tissue. Cancer Res 49:516–521, 1989.
341. Ghirlanda G, Uccioli L, Perri F, Altamonte L, Bertoli A, Manna R, Fiati L, Greco AV: Epidermal growth factor, somatostatin and psoriasis. Lancet 1:65, 1983.
342. Setyono-Han B, Henkelman MS, Foekens JA, Klijn JGM: Direct inhibitory effects of somatostatin (analogues) on the growth of human breast cancer cells. Cancer Res 47:1566–1570, 1987.
343. Sluyser M: Steroid/thyroid receptor-like proteins with oncogenic potential: A review. Cancer Res 50:451–458, 1990.
344. Evans RM: The steroid and thyroid hormone receptor superfamily. Science 240:889–895, 1988.
345. Damm K, Thompson CC, Evans RM: Protein encoded by v-*erb*A functions as a thyroid-

hormone receptor antagonist. Nature 339:593–597, 1989.
346. Kakizuka A, Miller WH, Umesono K, Warrell RP Jr., Frankel SR, Murty VVVS, Dimitrovsky E, Evans RM: Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RARα with a novel putative transcription factor PML. Cell 66:336–374, 1991.
347. de Thé H, Lavau C, Marchio A, Chomienne C, Degos L, Dejean A: The PML-RARα fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. Cell 66:675–684, 1991.
348. Warrell RP Jr., Frankel SR, Miller WH Jr., Scheinberg DA, Itri LM, Hittelman WN, Vyas R, Andreeff M, Tafuri A, Jakubowski A, Gabrilove J, Gordon MS, Dimitrovsky E: Differentiation therapy with tretinoin (all *trans*-retinoic acid). N Engl J Med 324:1385–1393, 1991.
349. Graham ML, Krett NL, Miller LA, Leslie KK, Gordon DF, Wood WM, Wei LL, Horwits KB. $T47D_{co}$ cells, genetically unstable and containing estrogen receptor mutants, are a model for the progression of breast cancers to hormone resistance. Cancer Res 50:6208–6217, 1990.
350. Lees JA, Fawell SE, Parker MG: Identification of constitutive and steroid dependent *trans*-activation domains in the mouse estrogen receptor. J Steroid Biochem 34:33–39, 1989.
351. Moncharmont B, Ramp G, DeGoeij CCJ, Sluyser M: Comparison of estrogen receptors in hormone-independent and hormone-dependent Grunder strain mouse mammary tumors. Cancer Res 51:3843–3848, 1991.
352. Fuqua SAW, Fitzgerald SD, Chamness GC, Tandon AK, McDonnell DP, Nawaz Z, O'Malley BW, McGuire WL: Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res 51:105–109, 1991.
353. Scott GK, Kushner P, Vigne J-L, Benz CC: Truncated forms of DNA-binding estrogen receptors in human breast cancer. J Clin Invest 88:700–706, 1991.
354. Fuqua SAW, Fitzgerald SD, Allred DC, Elledge RM, Nawaz Z, McDonnell DP, O'Malley BW, Greene GL, McGuire WL: Inhibition of estrogen receptor action by a naturally occurring variant in human breast tumors. Cancer Res 52:483–486, 1992.
355. Schmitt M, Janicke F, Graeff H: Tumour-associated fibrinolysis: The prognostic relevance of plasminogen activators uPA and tPA in human breast cancer. Blood Coag Fibrin 1:695–702, 1990.
356. Liotta LA, Steeg PS, Sltler-Stevenson WG: Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. Cell 64:327–336, 1991.
357. Bassett P, Bellocq JP, Wolf C, Stoll I, Hutin P, Limacher JM, Podhajeer OL, Chenard MP, Rio MC, Chambon P: A novel metalloprotease gene specifically expressed in stromal cells of breast carcinomas. Nature 348:699–704, 1990.
358. Steeg PS, Bevilaqua G, Kopper L, Thorgeirsson UP, Talmudge JE, Liotta LA, Sobel ME: Evidence for a novel gene associated with low tumor metastatic potential. J Natl Cancer Inst 80:200–204, 1988.
359. Rosengard AM, Krutzsch HC, Shearn A, Biggs JR, Barker E, Margulies IMK, King CR, Liotta LA, Steeg PS: Reduced nm23/*awd* protein in tumor metastases and aberrant Drosophila development. Nature 342:177–180, 1989.
360. Wallet V, Mutzel R, Troll H, Barzu O, Wurster B, Vernon M, Lacombe MA: Dictyostelium nucleoside diphosphate kinase highly homologous to nm23 and proteins involved in mammalian tumor metastases and Drosophila development. J Natl Cancer Inst 18:1199–1202, 1990.
361. Leone A, Flatow U, King CR, Sandeen MA, Margulies IMK, Kiotta LA, Steeg PS: Reduced tumor incidence metastatic potential and cytokine responsiveness metastatic potential and cytokine responsiveness of nm23-transfected melanoma cells. Cell 65:25–35, 1991.
362. Bevilacqua G, Sobel ME, Liotta LA, Steeg PS: Association of low nm23 RNA levels in human primary infiltrating ductal breast carcinomas with lymph node involvement and

other histopathological indicators of high metastatic potential. Cancer Res 49:5185–5190, 1989.
363. Henessy C, Henry JA, May FEB, Westley BR, Angus B, Lennard TWJ: Expression of the antimetastatic gene nm23 in human breast cancer: An association with good prognosis. J Natl Cancer Inst 83:281–285, 1991.
364. Stahl JA, Leone A, Rosengard AM, Porter L, King CR, Steeg PS: Identification of a second human nm23 gene, nm23-H2. Cancer Res 51:445–449, 1991.
365. Lee SW, Tomasetto C, Sager R: Positive selection of candidate tumor-suppressor genes by subtractive hybridization. Proc Natl Acad Sci USA 88:2825–2829, 1991.
366. Chen L-C, O'Bryan J, Smith HS, Liu E: Isolation of a matrix gla protein in breast carcinoma cells by differential cDNA cloning. Oncogene 15:1391–1395, 1990.
367. Cropp CS, Lidereau R, Campbell G, Champene MH, Callahan R: Loss of heterozygosity on chromosomes 17 and 18 in breast carcinoma: Two additional regions identified. Proc Natl Acad Sci USA 87:7737–7741, 1990.

3. Oncogenes in human lung cancer

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Introduction

While the last 15 years have not yielded substantial improvements in the diagnosis and treatment of lung cancer, this same period has experienced an extraordinary expansion of our understanding of cancer initiation and progression. These investigations have provided strong support for a multistep mechanism of tumor induction and progression [1,2] that previously could only be inferred from experimental animal models and from epidemiologic analyses. This recent work has also revealed that, in many cases, the structural alterations of the same gene products (for example, *myc* and *ras* or p53 and *Rb* genes) might be critical steps in the genesis of a wide range of adult tumors of differing histologic origins. These observations blur the distinction for investigators focusing on specific cancer models, while at the same time offer the hope that advances in the understanding and treatment of any particular tumor type will have far broader implications. In addition, now that the genetic basis for lung cancer has been well established, we are witnessing a resurgence of interest in mechanisms of specific environmental carcinogens as tools for a new generation of cancer prevention strategies [3]. There is currently no doubt that the use of tobacco products is the single most important (although not the only) causative factor in the development of lung cancer [4,5], and as these patterns of abuse spread there will be a projected worldwide increase in the incidence of lung malignancies paralleling that seen in North America and Europe over the last 50 years. Therefore, the challenge in the immediate future will be to apply the molecular clues obtained from the laboratory into effective preventive and therapeutic applications. The purpose of this chapter is to review some of the more important experiments concerning the molecular genetics of lung cancer, focusing on the identification (and implications) of somatic mutations in a limited number of cellular genes referred to as either dominant or recessive oncogenes.

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Small cell lung cancer

Lung cancer is the most common cause of cancer death in the United States, with a predicted incidence of 150,000 new cases in 1991 [6]. These tumors have been conveniently separated into two major categories (designated as small cell or non-small cell lung carcinoma) on the basis of their clinical behavior and their histologic appearance under light microscopy (Table 1). Small cell lung cancer constitutes about 25% of all cases of lung cancer and is an aggressive lung tumor with a high propensity for disseminated spread throughout the body. As a result, surgical resections of this tumor are rarely clinically indicated, limiting the ability of the investigator to obtain primary tumor material. Although these tumors are initially sensitive to cytotoxic chemotherapy and/or radiation therapy, with a reduction in tumor volume seen in almost all treated patients, the disease usually recurs, at which time it is usually refractory to any further treatments. In retrospect, the appearance of this tumor as a 'small cell' with pyknotic nuclei ('oat cell' or 'lymphocytic' appearance) is likely a crush artifact from the bronchoscopic biopsy, while well-preserved tissue sections show larger epithelial cells with a fine nuclear chromatin pattern [7]. Nonetheless, the histologic appearance of small cell lung cancer is characteristic and remains the only accepted method to establish the diagnosis.

One of the major advances in the study of the biology of lung tumors was

Table 1. Characteristics of lung cancer

	SCLC	NSCLC
Clinical		
Method of diagnosis	Light microscopy	Light microscopy
Distribution of cases	25%	75%
Primary treatment	Nonsurgical	Surgical
Dissemination at diagnosis	Frequent	Less frequent
Paraneoplastic syndromes: ectopic peptide secretion	Common	Uncommon
Biochemical		
Growth in tissue culture	Nonadherent clusters	Adherent monolayer
Neuroendocrine markers		
L-dopa decarboxylase		
Neuron-specific enolase		
Creatine kinase BB	Common	Rare
Chromagranin		
Neurosecretory granules		
Peptide secretion		
Arginine vasopressin		
Atrial natriuretic factor	Present	Absent
Gastrin releasing peptide		
EGF receptors	Rare	Present

[a] SCLC-small cell lung cancer; NSCLC-non-small cell lung cancer.

the ability to generate a series of continuous cell lines derived from biopsies of patients with lung cancer [8,9]. The cell lines obtained from small cell carcinoma samples characteristically grow as tight clusters of 20 to several hundred cells that nonadherently float in culture medium, making it difficult to obtain viable single cell suspensions. Early biochemical studies confirmed the observation that small cell lung cancer is a neuroendocrine tumor characterized by neurosecretory granules and the production of a cassette of neural specific enzymes and peptides, including dopa-decarboxylase, neuron-specific enolase, the brain isoenzyme of creatine kinase, chromogranin A, bombesin-like immunoreactivity or gastrin releasing peptide, ACTH, arginine vasopressin, atrial natriuretic factor, and others [8,10–13]. The significance of the production of large amounts of functionally active bioenergetic enzymes (i.e., enolase and creatine kinase) is unknown; however, the expression of other products, such as gastrin-releasing peptide, has been speculated to play a mitogenic role in an autocrine feedback loop mechanism [14]. In summary, these immortalized cell lines have continued to serve as an invaluable resource for biochemical and subsequent cytogenetic studies that have ushered in the first investigations on genetic alterations in lung cancer.

Non-small cell lung cancer

Non-small cell lung cancer comprises all other types of lung cancer and, as a result, is a collection of at least four histologic types: squamous cell carcinoma, bronchioloalveolar and adenocarcinoma, large cell carcinoma, and undifferentiated lung carcinoma. Although these tumors are less likely than small cell carcinomas to undergo early dissemination, they are characterized by aggressive local spread and are less responsive to treatments with cytotoxic chemotherapy or radiation therapy. As a result, patients are more likely to undergo an attempt at surgical resection, allowing the collection of primary tumor material for laboratory investigations. In addition, tumorigenic cell lines can also be derived from these samples and such cultures typically grow readily as an adherent monolayer with fibroblastlike morphology.

Although this classification of small cell vs. non-small cell has been useful for both pathologists and clinicians, it incorrectly implies an understanding of the cell of origin of these tumors. Intuitively, it is believed that small cell carcinomas arise from rare cells with neural APUD (amine precursor uptake and decarboxylation) features that are buried in the bronchial mucosa, while non-small cell carcinomas arise from varying other precommitted bronchial epithelial cells. For years, however, pathologists have observed lung tumors characterized by admixtures of both small cell and non-small cell histology. In addition, about 10–15% of non-small cell tumors exhibit features of neuroendocrine differentiation, while a similar proportion of small cell tumors express no neuroendocrine products [15]. These observations have

raised the hypothesis ('unitarian theory' of lung cancer) of a single progenitor cell that gives rise to varying types of lung tumors [16]. As will be discussed below, however, the molecular genetic analysis of small cell and non-small cell tumors will reveal as many similarities as differences between the two histologic types, and at least for the time being we will continue to use the same pathology-based classification system.

Recessive oncogenes in lung cancer

An elusive lung cancer susceptibility gene on chromosome 3p. An early clue for the possibility of a lung cancer susceptibility gene was suggested in 1982 in a cytogenetic analysis of a series of small cell lung cancer cell lines. Although all karyotypes demonstrated multiple alterations, the most consistent abnormality was a deletional event on the short arm of chromosome 3 [17,18], and more precise cytogenetics allowed the smallest consensus deletion to be mapped to the region of 3p14-3p24. This finding was subsequently tested by restriction fragment length polymorphism (RFLP) analyses of both primary small cell and non-small cell carcinomas [19–23]. These investigators verified that there was evidence at the molecular level for DNA loss on one allele of chromosome 3p in >90% of informative cases of small cell lung cancer, while in non-small cell lung cancer a similar allelic loss was observed in approximately 50% of samples tested [21,22]. The consistent finding of tumor-specific chromosomal deletions in this region led to the hypothesis that a lung cancer susceptibility gene mapped to this area. Using the retinoblastoma gene as a paradigm (see below), this hypothesis proposed that inactivation of this chromosome 3p cellular gene would result in deregulated cellular proliferation and would play an important role in the multistep pathway to frank neoplasia. Attempts to identify the putative recessive oncogene on chromosome 3p, however, have met with little success to date.

One approach to identify a putative '3p tumor-suppressor gene' has been to examine interesting genes that have previously been characterized and mapped to the vicinity of this region on chromosome 3p. Several candidate genes have been studied, including the thyroid hormone receptor (c-*erb*A-β), a retinoic acid receptor (*rar*β, previously designated *hap*1), and a transcribed gene of unknown function at the DNF15S2 locus of chromosome 3p21 [24–29]. Although each of these genes undergo allelic loss in most (but not all) tumor samples from patients with small cell lung cancer, evidence for homozygous mutations and/or loss of wild-type protein function has not been reported for these cases. Recently, a gene encoding a tyrosine phosphatase (PTP-gamma) has been proposed as a candidate for the 3p tumor suppressor gene [30]. This gene maps to chromosome 3p21 and is one of a family of phosphatase enzymes that helps mediate information signalling pathways in the cell. In 5 of 10 primary non-small cell lung cancer samples tested, there was evidence for allelic loss of PTP-gamma, and in 3

of these 5 cases there was retention of loci flanking the PTP-gamma gene. Although this finding suggested the possibility of loss of a discrete portion of chromosome 3p at or very near the PTP-gamma gene, homozygous abnormalities have not been reported as yet to indicate that this gene is specifically targeted for inactivation in lung cancer.

The search for a lung cancer 3p gene has outlined several difficulties: First, prior work with the retinoblastoma gene demonstrated that potential tumor suppressor genes might be inactivated by subtle deletions or point mutations that could escape detection by routine DNA or RNA blotting techniques [31], emphasizing the meticulous work that must be applied in the analysis of candidate genes. Second (and more importantly) lung cancer does not have a clear familial predisposition with well-recognized kindreds available for the powerful gene linkage analyses that have aided in the cloning of other human disease genes, such as the cystic fibrosis or neurofibromatosis-1 genes [32,33]. Although recent epidemiologic studies have suggested an inherited predisposition in certain populations with lung cancer [34], the significance of this contribution appears too small for practical applications. In contrast, familial renal cancer and renal tumors within the von Hippel-Lindau syndrome have been genetically linked to chromosome 3p, and large-scale efforts are underway to delineate a manageable stretch of DNA to begin searching for transcribed genes [35–38]. Additional evidence supporting the presence of a functional renal cancer gene was the ability to reverse the tumorigenicity of a renal tumor cell line with the introduction of chromosome 3 material by micro-cell fusion techniques [39]. These observations have, optimistically, suggested the possibility that the renal cancer tumor suppressor gene may also be the 3p gene targeted in lung cancer. Alternatively, translocations and RFLP analyses from renal tumors has noted DNA loss near chromosome band 3p14 (near the centromere) [35,37], while linkage analysis and other studies have placed the gene closer to the telomere [36,38].

Similar inconsistencies in defining a minimum consensus deletion by RFLP analyses have also been observed in lung cancer. This may be explained by the presence of a known fragile site at 3p14 that is associated with increased breakage and sister chromatid exchange in the peripheral blood and bone marrow of young cigarette smokers [40], or these findings may suggest that there are multiple recessive oncogenes on chromosome 3p. In fact, in addition to lung cancer and renal cancer, several other tumors have also been associated with deletions of chromosome 3p, including cervical [41] and breast cancers [42]. Currently, vigorous attempts are underway in several laboratories to clone the predicted 3p lung cancer gene using systematic methods of molecular biology, including subtractive libraries, chromosome-specific libraries, and further analysis of current candidate genes and loci. These efforts, in cooperation with an international human genome effort and the mapping of human chromosome 3, will address these questions in the near future.

The *Rb* gene

Overview. In 1986, a gene located at chromosome 13q14 was identified as the candidate locus that conferred susceptibility to the development of familial and sporadic retinoblastoma tumors [43]. The existence of this gene, named the retinoblastoma susceptibility (*Rb*) gene [43–45], was predicted by Knudson in 1971 on the basis of a mathematical analysis of epidemiologic data [46]. He postulated that two discrete genetic mutations would be necessary to inactivate both alleles of a recessive oncogene, resulting in the development of retinoblastoma tumors (the 'two-hit' hypothesis). This hypothesis was initially supported by the detection of a specific chromosomal deletion (13q14) in certain patients with familial retinoblastoma [47] and subsequently was confirmed with careful RFLP analysis of germline and tumor material from affected patients [48]. Evidence for the authenticity of this gene as a tumor suppressor gene came primarily from the observation that (1) some tumor samples contained interstitial deletions within the *Rb* gene, suggesting that the mutation was targeting the *Rb* and not adjacent loci; (2) the RB protein product is inactivated in all retinoblastoma tumors examined to date; and (3) suppression of tumorigenicity was observed in a retinoblastoma cell lines with the introduction of the *Rb* gene [49,50].

The RB gene product is a nuclear phosphoprotein that migrates on SDS-PAGE as a series of closely spaced bands of approximately 110–115 kDa, with the more slowly migrating bands representing differentially phosphorylated species [51]. Since the absence of RB protein was associated with deregulated cell growth, an early hypothesis for RB function was that it somehow regulated cell cycle events. This hypothesis was greatly strengthened by the observation that the phosphorylation state of the RB protein was tightly synchronized with specific stages of the cell cycle [52–57]. For example, resting cells (G_0) or cells in early G_1 expressed largely unphosphorylated protein, while RB became progressively phosphorylated during S phase and early mitosis. Further, the recognition that RB is phosphorylated by the kinase system (cyclin:cdc2/cdk complex), which had previously been shown to regulate motosis and meiosis checkpoints in all eukaryotic cells, unequivocably links RB biochemistry with the cell cycle [58,59].

Related experiments has shown that the RB protein can bind specifically to the transforming proteins of at least three different classes of DNA tumor viruses (large T of SV40, E1A of adenovirus, and E7 of human papillomavirus) [60–62]. These unexpected interactions predicted that RB activity was normally modulated by cellular protein(s) and that these DNA tumor viruses had usurped this function to allow enhanced viral proliferation. A strategy based on this viral oncoprotein: RB interaction led to the identification of a growing set of cellular proteins [63–67] that specifically bind to RB: the transcription factor E2F, c-*myc*, and N-*myc* oncoproteins, and the retinoblastoma binding proteins 1 and 2 (RBP1 and RBP2). Of

interest, the viral large T antigen and the cellular E2F protein appear to bind preferentially to underphosphorylated RB [68]. This observation, as well as the association of unphosphorylated RB with quiescent or early interphase cells, has been interpreted to suggest that the underphosphorylated RB species are the 'active' growth suppressor molecules.

Lung cancer and *Rb*. Interest in the *Rb* gene and lung cancer was stimulated by ongoing research to identify and clone the putative 'chromosome 3p gene' discussed above. Review of cytogenetic data, however, revealed that numerous examples of deletions or unbalanced translocations involving the chromosome band 13q14 were also present in karyotypes of small cell lung cancer cell lines [69]. These had been previously overlooked due to the extensive aneuploidy found in these samples, and this suggests that additional marker chromosome abnormalities may yet be found (see below). Similarly, review of RFLP data from primary lung tumor samples demonstrated frequent loss of heterozygosity, not only on chromosome 3p, but also on 13q and 17p in small cell lung cancer [21]. Although small cell lung cancer had a completely different clinical presentation than retinoblastoma, these tumors, nevertheless, present similar histopathologic characteristics: They both exhibit features of neural differentiation, they grow nonadherently in cell culture as tight clusters of several hundred cells, and they both frequently amplify *myc* family genes [70–73]. Therefore, the *Rb* gene, which had recently been cloned, was a potential target for somatic mutations in small cell lung cancer.

Early DNA and RNA studies demonstrated evidence for structural mutations in 20% of small cell lung cancer samples tested and absent mRNA in approximately 40% of derived cell lines [69]. In addition, in each case where a matched primary tumor sample was available, the abnormality detected was identical to that observed from the derived cell line. Further studies of RB protein revealed that the majority of small cell lung cancer samples that expressed apparently normal mRNA had no detectable RB protein [74,75]. In addition, several examples of mutant RB proteins were found as a result of subtle structural mutations, generally single point mutations. To date these have included aberrant deletion of nucleotide sequences from exons 16, 21, or 22, yielding stable, but truncated, proteins with intact amino- and carboxyl-terminal residues [74,76]. Some of these same abnormalities have been observed in other tumor types, such as a deletion of exon 21, which was reported in both a bladder tumor [77] and a prostate tumor [78], while an example of deletion of exon 22 has also been seen in a case of non-small cell lung cancer [79]. Another interesting mutation identified in small cell lung cancer was a single nucleotide change in exon 21 that led to missense mutation (cys^{706} to phe^{706}) [80]. Each of these in vivo RB protein mutants share the same phenotype: They are defective in viral oncoprotein binding (and, therefore, also defective in their ability to bind to the corresponding cellular binding proteins) and in phosphorylation. Since the mutations do

not directly affect a cluster of serine and threonine residues that are the presumed sites of phosphorylation, these mutations [58], instead, appear to block a specific conformation required for protein binding and phosphorylation. In fact, a series of in vitro generated mutants have mapped the domain on RB for oncoprotein binding [81–83], and all mutants that have lost binding function are also defective in phosphorylation, although the reverse may not necessarily be true [84]. The in vivo RB mutants, therefore, are presumed to be inactive ('loss of function mutants'), and this is consistent with their inability to transform primary rat embryo cells by themselves or in cooperation with an activated *ras* gene [79]. Further, simultaneous expression of both wild-type and mutant protein has not been observed in any tumor samples from either retinoblastoma or adult solid tumors, implying that homozygous genetic alterations with complete wild-type inactivation has occurred in all cases.

In summary, essentially all small cell lung cancer samples have absent or aberrant RB protein expression. In contrast, only about 15% of non-small cell lung cancer tumors have inactivated RB function [69,85]. This lower frequency of *Rb* inactivation is seen in a wide range of many adult tumors, such as bladder, prostate, breast, hematologic, and other tumors [74,86,87]. The biologic significance of the *Rb* gene as a high-frequency target in small cell lung cancer, but a lower frequency target in other common tumors, is still uncertain.

Another unresolved issue is why germline mutations in the *Rb* gene (as seen in patients with familial retinoblastoma) are not associated with a high risk for small cell lung cancer. Prior studies of retinoblastoma survivors had not identified small cell lung cancer as a common secondary tumor. A recent analysis, however, of subjects who were identified clinically or epidemiologically as carriers of a mutant *Rb* allele reported a 15-fold increase risk of lung cancer in this group, which appeared predominantly to be small cell lung cancer [88]. If this association is correct, then it appears that these carriers are at a significantly higher risk for small cell lung cancer, but nonetheless still require time to stochastically accumulate additional genetic lesions, such as p53 mutations, chromosome 3p deletions, and others. Removal of incriminating carcinogens, therefore, is especially important for these individuals.

Reintroduction of the *Rb* gene in a variety of $Rb^{(-/-)}$ cell lines, such as retinoblastoma [49], osteosarcoma [49], prostate [78], and bladder cancer [89] derived lines, has resulted in complete or partial suppression of tumorigenicity when measured as the ability to form subcutaneous tumors in athymic mice. Preliminary work in lung cancer also show suppression of tumorigenicity in nude mice in an $Rb^{(-/-)}$ non-small cell lung cancer cell line that was stably transfected with an *Rb* gene under a cytomegalovirus promoter [79]. Recently, however, a conflicting study reported no suppression of tumorigenicity in a retinoblastoma cell line, despite stable exogenous expression of RB protein [90]. This emphasizes the fact that the

primary role RB plays in cell physiology has not been identified, which necessarily limits the ability to assay for activity with gene replacement.

The identification of two-hit mutational inactivation of the *Rb* gene in small cell carcinoma was the first example of a recessive oncogene clearly involved in the pathogenesis of lung cancer. This finding, along with the subsequent recognition of p53 mutations, confirmed the multihit genetic etiology of lung cancer. This insight will undoubtedly lead to attempts to develop new strategies for prevention, early detection, and treatment. Recently, several studies have correlated RB protein immunohistochemistry tissue staining with clinical prognosis, and one report observed a direct correlation with reduced RB tissue immunostaining and a more aggressive clinical course in patients with malignant sarcoma [91]. Another study observed reduced expression of RB protein in a higher fraction of tumor specimens with advanced-stage disease (stage III and IV) than earlier stages [92]. These authors have suggested that reduced/loss of RB is associated with more aggressive clinical disease. Although promising, difficulties in interpreting protein staining data arise from the uncertain biologic significance of reduced RB levels (gene inactivation had previously been defined as absent or aberrant protein) and because many mutant proteins react with commercially available antisera. Additional studies will continue define the proper role for this new technology in clinical practice.

The p53 gene

Overview. The p53 gene encodes a 53-kDa nuclear protein that localizes to chromosome 17p13. It was originally identified as a host cellular protein that bound to the large T viral antigen of animal cells infected with simian virus 40 (SV40) [93–95], and in those early reports it was already speculated that the large T antigen might exert its transforming effect by binding to and, therefore modulating, the activity of the cellular p53 gene [93]. Several years later, however, it was classified as a 'dominant transforming gene' on the basis of elevated protein levels in many tumors and the ability of its cDNA to transform primary rat embryo fibroblasts when transfected in cooperation with an activated *ras* gene [96–99]. In 1988, however, it was appreciated that all transforming clones of p53 had undergone activating somatic mutations, while, in contrast, wild-type p53 appeared to exert a phenotype of growth suppression when transfected into immortalized cell lines [100–102]. In addition, the elevated steady-state levels of p53 observed in many tumors was the result of mutations that markedly increased the protein half-life, and the detection of increased protein levels on immuno (Western)-blotting is one of the methods presently used to screen samples for the presence of activating p53 mutations.

It is now recognized that the p53 gene is the most frequently mutated gene in human cancers and, consequently, plays an important role in the transformation of a wide range of distinct tumors. Although the mechanism

of action of p53 is still unknown, it appears to play an important multifunctional role and has been implicated in both DNA replication and as a potent transcriptional activator [103,104]. However, a strong argument against p53 playing an essential role in normal cellular physiology was the recent report of the normal development of mice with an engineered knockout of the p53 gene [105]. Mice heterozygous for a germline mutation in their p53 gene (generated by homologous recombination into embryonal stem cells) were inbred to create offspring that were homozygous for mutant p53 alleles. Although these mice appeared phenotypically normal, these mutations did confer a markedly increased susceptibility to a range of different tumors [105]. This finding is consistent with human in vivo data that suggest p53 is not critical for cell growth and differentiation but rather subserves an important role in safeguarding against tumor formation.

There are two related hypothesis to explain the effect of mutant p53 on the formation of tumors in human cells. One hypothesis predicts that the mutant protein can act to bind to wild-type p53 molecules, thus subverting the normal function of p53 in the nucleus [100–102]. This 'dominant-negative' mechanism has considerable support from in vitro experiments that demonstrate that the p53 gene product can undergo homooligomerization, forming a protein complex that may be necessary for its functional activity. In addition, wild-type p53 could suppress the transforming activity (in rodent cells) of mutant p53 in a dose-dependent manner. Arguments against an important role for a dominant-negative mechanism in human cancer, however, was the observation that wild-type and mutant p53 protein have not been observed to be coexpressed in the same human tumor sample. In addition, when subjects carrying a germline mutation of the p53 gene (i.e., cancer-prone families, such as the Li-Fraumeni syndrome) develop a malignant tumor, the wild-type allele has invariably been inactivated [106]. These observations resemble the pattern seen with the *Rb* gene, suggesting that p53 may also behave as a recessive oncogene. Regardless, wild-type p53 functions as a potent growth and tumor suppressor when transfected into human or rodent cell types [100], offering the hope that this activity might be exploited in future clinical studies.

Lung cancer and p53. Earlier RFLP analyses had implicated tumor-specific loss of heterozygosity on chromosome 17p in both lung cancer and colon cancer [21,107]. Shortly afterward it was demonstrated that the gene on 17p targeted for allele loss in colon cancer was the p53 gene, which had frequently acquired a somatic mutation in the open reading frame of the remaining allele [108]. Similar investigations of lung cancer revealed that essentially all (70–100%) small cell lung cancers have acquired mutations, while approximately 40% of non-small cell lung cancers have evidence for p53 mutations [109–113]. In these resected specimens, the presence of p53 abnormalities was not consistently correlated with tumor stage or histology, although there was a relationship between an increased smoking history and

the frequency of somatic mutations [114]. When material was available for analysis, the identified mutations were not found in adjacent normal lung from the same patients, arguing against a 'field defect' phenomena in these tumors. The high frequency in small cell lung cancer of p53, as well as *Rb* abnormalities, suggests that these genetic alterations are early events in the tumorigenic pathway of this disease. Unfortunately, lung cancer does not have a readily available 'premalignant' model to test this hypothesis, such as the benign and dysplastic colonic polyps observed in the model of progression toward colon cancer. In colon cancer it has been argued that p53 mutations are late, and perhaps rate-limiting, genetic events for the development of the aggressive, malignant phenotype [115]. This observation appears to be true for bladder and primary brain tumors as well [116,117], although clonal abnormalities of p53 have been reported in the premalignant esophageal epithelia referred to as Barrett's esophagus [118]. The development of a model for premalignant respiratory epithelia will be a useful tool to help address these important issues.

The in vivo p53 mutations identified in the different types of human tumors seem to cluster around region of the open reading frame that have been conserved across species [100]. More interestingly, the specific types of mutations appear to be loosely correlated with specific types of carcinogen exposure. For example G to T transversions of p53 predominate in tobacco-related tumors, while G to A transitions are more common in colonic tumors, and mutations from thymidine dimers occur in ultraviolet light-associated skin cancers [119,120]. Although these finding are not surprising, they, nonetheless, add further validity to a model of human tumorigenesis that is developing and suggest an objective assay to attempt to identify exogenous and endogenous carcinogens.

Table 2. Cytogenetic abnormalities in lung cancer[a]

SCLC	NSCLC
3p14-3p24	3p21-3p25
13q14	3p14
17p	i(3q)
11p	11p
1p	17p
5q	1q1-1q3
	9p
	17q
	19q13
	5q1
	7q

[a] Selected listing of observed cytogenetic abnormalities in small cell lung cancer [17,18,121,122] and non-small cell lung cancer [123,124].

Other recessive oncogenes. The accuracy with which cytogenetics and RFLP analyses predicted the presence of tumor suppressor genes at the 13q (*Rb*) and 17p (p53) regions suggests that the presence of nonrandom, tumor-specific evidence for allele loss is an important clue for the identification of additional recessive oncogenes. Therefore, in addition to 3p abnormalities, many other chromosomal loci have been identified as frequent sites for gross structural mutations in lung cancer [17,18,121–124] (Table 2), suggesting that many of these additional sites may also harbor novel genes mediating normal growth and development for pulmonary tissues.

Activation of dominant oncogenes in lung cancer

myc. Since the first recognized oncogenes belonged to a category referred to as 'dominant oncogenes,' it was only natural that the earliest work on the genetic etiology of lung cancer involved these genes. Homogeneously staining regions (HSRs) and double-minute chromosome fragments had been observed in the karyotypes of small cell lung cancer for several years. Prior work had shown that these staining patterns often represent regions of gene amplification, and in 1983 amplification of the c-*myc* gene was reported in 7 out of 18 small cell lung cancer cell lines [71]. In addition, it was believed that those lung tumor cell lines with amplified DNA and over-expressed c-*myc* mRNA had a characteristic 'variant' phenotype with a more aggressive growth pattern and a less differentiated appearance [71]. Further, the transfection of a c-*myc* gene, under the control of viral promoter, into a 'classic' small cell lung cancer cell line resulted in an altered phenotype resembling a 'variant' morphology [125]. Cytogenetic studies of these cell lines showed that translocations involving the chromosome band 8q24 are not characteristically seen in these tumors, in contrast to the pattern of *myc* activation seen in certain human lymphoid tumors. When additional lung tumor samples were tested for the presence of *myc* amplification, however, several cell lines exhibited novel hybridizing bands that could not be explained on the basis of a rearrangement of the c-*myc* gene. These bands represented amplification of two other related *myc* family genes: the N-*myc* gene, which had previously been cloned from neuroblastoma tumor tissue [72], and the L-*myc* gene [73].

In summary, overexpression (with or without *myc* gene amplification) of one of the related *myc* oncogenes occurs in approximately 10–40% of tested small cell lung cancer primary tumors and derived cell lines [126–129]. Variabilities in the reported frequencies of *myc* amplification in lung cancer, however, is partly due to misinterpretation of moderate increases in band intensity on Southern blotting due to chromosome hyperdiploidy as representing true gene amplification. Since the presence of *myc* amplification was reported to be higher in cell lines than in primary tumor samples, it was believed that this genetic event occurred late in tumor progression pathways.

However, other studies have observed that both cell lines and corresponding primary tumor (when available) were concordant with *myc* amplification [126–129], although the frequency reported for primary tumors was 5–15%. These subsequent studies have not supported the correlation of *myc* amplification in small cell lung cancer with a 'variant' cell line morphology, but it appears that prior exposure to cytotoxic chemotherapy may be an important stimulus [127–129].

Whereas 8% (3 of 40) specimens from untreated SCLC patients demonstrated amplified *myc* family DNA, 28% (19 of 67) of the tumor specimens from patients previously treated with combination chemotherapy demonstrated such *myc* amplification [129]. In addition, there may be a differential effect from specific chemotherapy agents, as c-*myc* amplification may occur less frequently in patients treated with the more recent regimen of etoposide/cisplatin [129]. Although chromosomal rearrangement of the *myc* genes are not believed to be an important mechanism of *myc* activation in carcinomas, two independent small cell lung cancers were identified with an intrachromosomal rearrangement fusing an amplified L-*myc* gene with a previously unknown transcribed gene (designated *rlf*) [130]. Since the *rlf* gene, which is located within 800 kb of the L-*myc* gene, contributes amino terminal sequences to the chimeric polypeptide, this alteration may play a role in the development of these tumors [131].

Since amplification of N-*myc* has been associated with a more aggressive behavior and inferior survival in pediatric neuroblastoma tumors [132], several reports have investigated the correlation of *myc* amplification in lung cancer with patient survival. These studies have shown a small, but statistically significant, decrease in survival time in patients whose tumors exhibited c-*myc* DNA amplification [127–129], while no clinical correlation was observed with amplification of N-*myc* or L-*myc* in these samples. Another study, however, did detect increased expression of N-*myc* by in situ hybridization techniques in biopsies of patients with poor-prognosis small cell lung cancer [133]. Since human tumors can exhibit a range of sensitivity to ionizing radiation delivered in vivo or in vitro, investigators have observed the ability of certain oncogenes, such as *myc* or *ras*, to modulate radiosensitivity [134,135]. Studies on different xenografts of SCLC tumors from the same patient, however, showed varying radiosensitivity with no correlation to oncogene expression [136].

The *myc* genes share many structural and functional similarities, including several domains of striking amino acid identity throughout their respective encoded open reading frames [137]. The proteins encoded by these genes share extensive homology, including a basic domain helix-loop-helix (bHLH) and leucine zipper (LZ) motifs in the carboxyl terminus of the protein. These regions have been identified as the binding site of a cellular protein (designated Max for *myc*-associated protein X) that confers a specific DNA binding activity to the resulting complex [138]. The recognition of this DNA binding activity to the sequence CAGGTG [139] is the first step to identify

the target genes regulated by *myc*:Max, which should then help unravel the role *myc* plays in cell growth and differentiation. In addition, the amino-terminal region of *myc* has been implicated in the binding of the retinoblastoma protein [66]. If this interaction occurs in vivo, this surprising finding raises many more questions about the role *myc* might play in cell-cycle events and as an early participant in tumor formation. In particular, since retinoblastoma (and small cell lung) tumors frequently amplified and overexpressed the *myc* oncogenes, an early hypothesis for RB function was that it acted to suppress *myc* expression [70]. Recent work demonstrating that RB can suppress transcription of early response genes, such as *fos* [140] and *myc* [141], may support this model.

Although *myc* genes share many similarities, they have a divergent pattern of tissue expression that has not yet been explained [142]. In addition, although c-*myc* and N-*myc* are highly tumorigenic in cooperation with an activated *ras* in rat embryo cells, L-*myc* is only weakly tumorigenic [143]. Finally, the L-*myc* gene undergoes a complex pattern of alternative intron splicing and polyadenylation site utilization that generates a family of mature mRNA that have the potential to encode a truncated protein with a novel carboxyl terminus [137]. All these findings suggest that these genes may encode distinct functions as well. Studies of the L-*myc* gene in lung cancer have largely focused on an RFLP due to a polymorphic *Eco*R1 site within intron 2 [134]. These reports have noted an association of a metastastic phenotype with the presence of the smaller *Eco*R1 band (6.6 kb band) and not the larger 10.0-kb polymorphic [144]. Subsequent reports, however, have not confirmed this association [145,146], and the biologic significance of this finding is still under study.

ras. The *ras* oncogenes are members of a supergene family that has been conserved from yeast to humans. There are three mammalian *ras* genes — H-*ras*, K-*ras*, and N-*ras* — which were initially identified by their ability to transform animal cells in culture in a dominant mechanism [147]. These genes are now recognized to encode a 21-kDa cytoplasmic protein, which can tightly bind guanine nucleotides (G-proteins) and which are believed to function as mediators of information (signals) across membranes. Their conversion into transforming proteins, as a result of acquired missense mutations, reduces their ability to metabolize guanine nucleotides, and this is hypothesized to 'lock' the protein in a conformation signaling growth stimulation [147]. Since these mutations are generally restricted to codons 12, 13, or 61, it has been possible to easily screen a variety of human tumors for their presence. These analyses have identified activated *ras* genes in a variety of human tumors, including lung, bladder, colon, breast, kidney, pancreas, liver, ovary, stomach, hematologic tumors, mesenchymal tumors, and others, and their overall incidence is estimated at approximately 10–15% [147]. The likelihood of finding a *ras* mutation, however, varies greatly among tumor types, with very low frequencies observed in breast cancer,

while about 50% of colon cancer and greater than 90% of exocrine pancreas tumors have mutations.

In lung cancer, for unknown reasons, the K-*ras* allele appears to be preferentially targeted, and activated *ras* genes can be found in approximately 30% of non-small cell lung cancers [148–151]. One group of studies found an overrepresentation of the adenocarcinoma subtype with *ras* mutations [148,149], while involvement of all subtypes were observed in another report [151]. In contrast, 0 of 37 small cell lung cancer cell lines had *ras* mutations [151]. In non-small cell lung cancer, the majority of these tumors involved codon 12 of K-*ras* [149,151], predominantly with a G to T or A to T transversion. These nucleotide changes are similar to the type of mutations reported with inactivation of the *Rb* and p53 genes in samples of patients with lung cancer. These studies also showed a statistically significant correlation with heavy cigarette smoking and K-*ras* mutations compared to patients with tumors bearing wild-type *ras* sequences [149]. The metabolism of inhaled cigarette smoke results in its conversion to over 3000 different chemicals [5], and of these a group of polycyclic aromatic hydrocarbons, such as benzo(a)pyrene and dimethylbenzanthracene, have been especially implicated as carcinogens of occupational concern. In particular, experimental lung tumors or skin tumors in mice treated with benzo(a)pyrene have been associated with G to T mutations at codon 12 in the K-*ras* gene [152,153]. In summary, these findings are supportive evidence that chemical carcinogens can, in fact, accelerate in vivo the accumulation of mutations targeted to specific regulatory genes, thus resulting in neoplastic transformation.

Recent studies have proposed that the presence of a K-*ras* mutation is a negative prognostic factor in lung cancer. In a series of 69 patients with completely resected adenocarcinoma of the lung, 12 of 19 patients harboring a codon 12 mutation died in the follow-up period as compared with 16 of the remaining 50 patients [154]. Interestingly, even though they had manifested a poorer prognosis, the patients with K-*ras* mutations initially presented with a lower clinical stage and smaller tumor volume than those without K-*ras* mutations. Another recent study also demonstrated an inferior survival in patients with non-small cell lung cancer and *ras* mutations [155], further suggesting that there may be a biologic difference conferred by this mutation.

Other dominant oncogenes. Activation of several other dominant oncogenes have been shown in isolated reports. These have included evidence for amplification and overexpression of c-*erb*-1 (EGFR) and c-*erb*-2 (*neu*) [156] in non-small cell lung cancer, and c-*myb* [157] and c-*fms* [158] in small cell lung cancer. In addition, overexpression without gene amplification has been reported for c-*raf* [158] and c-*kit* [159] in small cell lung cancer. Until more work is done, however, the clinical and biologic significance of these isolated abnormalities is uncertain.

Summary

The rapid pace of research in the genetics of human cancer will predictably render any review of the topic out of date by the time of its publication. Prospects for the near future will likely include the identification of a chromosome 3p gene(s) linked with the development of familial renal cancer and, perhaps, also lung cancer. In addition, the availability from the Human Genome Project of an increasing number of well-characterized markers will accelerate the search for additional human recessive oncogenes.

Many questions still remain about the etiology of lung cancer and how to apply this information for patient care. For example, identification of the cell of origin for small cell and non-small cell lung cancers will facilitate our understanding of the development of these tumors and improve the possibilities for future preventive strategies. In addition, we now realize that these cancers arise from the sequential accumulation of multiple genetic mutations (Table 3; Fig. 1). Therefore, a central question is which of these targets are essential for the process of carcinogenesis, and whether there is a critical temporal order for this process with a defined premalignant phase in a discrete field of bronchial tissue. In addition, are there genetically

Table 3. Frequency of selected oncogene abnormalities in human lung cancer samples

	Allele loss at Chromosome 3p	Absent or aberrant Rb protein	Absent or aberrant p53 protein	Activated *ras*	Activated *myc*
SCLC	>90%	>90%	90%	0%	10–40%
Non-SCLC	50%	15%	50%	33%	

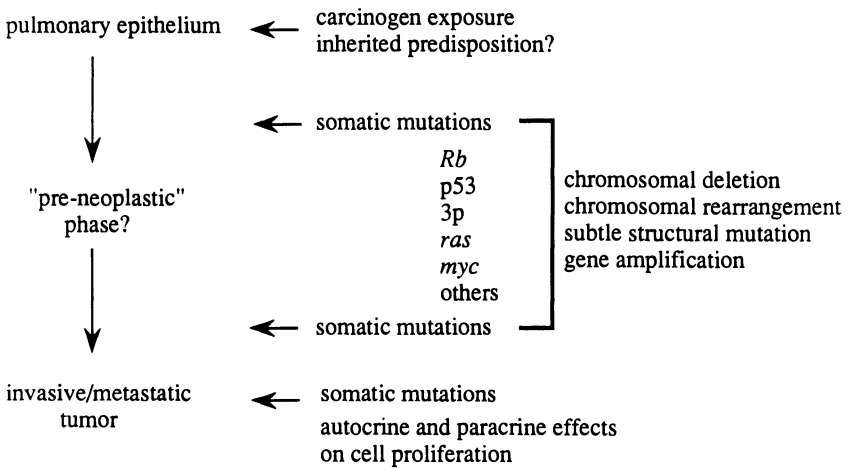

Figure 1. Multistep genetic model for lung cancer (modified from Kaye [160]).

inherited susceptibilities to the development of lung cancer (either directly or via variabilities in carcinogen metabolism) that could be accurately identified in the general population? Finally, is there a rate-limiting mutation and will the genetic correction of this defect suffice to restore growth regulation, or will the replacement of multiple gene products be required for tumor suppression?

We are already witnessing the beginnings of the use of molecular diagnostic markers as a research tool for assigning prognostic information. The expression of neuroendocrine markers in non-small cell lung cancer has recently been applied as an indicator of the potential response to combination chemotherapy [15]. Similar methods are being applied to the expression of tumor suppressor genes or the presence of somatic mutations in dominant oncogenes such as the *ras* gene. However, the clinical benefit of this prognostic information with currently available treatment programs is still uncertain. Reversion of tumorigenicity observed with the replacement of *Rb*, and particularly the p53 gene, has stimulated the development of animal models to test the feasibility of in vivo tumor suppression. Although the continuing development of viral vectors for effective transfer of other genes in pulmonary diseases, such as alpha-1 antitrypsin deficiency [161] or cystic fibrosis [162], makes this form of genetic therapy for lung cancer, a possibility for the future, safe, and efficient gene delivery for disseminated cancer does not seem practical with available technology. Perhaps the best hope for the early application of molecular oncology lies with research aimed toward the goal of prevention. As discussed above, patterns of mutations may reveal a fingerprint for endogenous and environmental mutagens and will put a growing focus on gene repair mechanisms. At a minimum, the public dissemination of the detailed genetic consequences of cigarette exposure (or other carcinogens) that has now become available should strengthen legislative/public resolve against these exposures [120].

References

1. Nowell PC: The clonal evolution of tumor cell populations. Science 194:23–28, 1976.
2. Land H, Parada L, Weinberg R: Cellular oncogenes and multistep carcinogenesis. Science 222:771–778, 1983.
3. Shields PG, Harris CC: Molecular epidemiology and the genetics of environmental cancer. JAMA 266:681–687, 1991.
4. Doll R, Peto R: The causes of cancer: Quantitative estimates of avoidable risks of cancer in the United States today. J Natl Cancer Inst 66:1191–1308, 1981.
5. United States Department of Health and Human Services: The health consequences of smoking: Cancer. A report of the Surgeon General, Office on Smoking and Health, Department of Health and Human Services, Rockville, MD, USA, 1982.
6. American Cancer Society: Cancer Facts and Figures. American Cancer Society, 1599 Clifton Road, NE, Atlanta, GA 30329, USA, 1989, p 9.
7. Matthews MJ, Gazdar AF: Pathology of small cell carcinoma of the lung and its subtypes. A clinico-pathologic correlation. In: Livingston RB (ed): Lung Cancer 1, Martinus

Nijhoff, Boston, 1981, pp 283–306.
8. Carney DN, Gazdar AF, Bepler G, Guccion JG, Marangos PJ, Moody TW, Zweig MH, Minna JD: Establishment and identification of small cell lung cancer cell lines having classic and variant features. Cancer Res 45:2913–2923, 1985.
9. Gazdar AF, Carney DN, Nau MM, Minna JD: Characterization of variant subclasses of cell lines derived from small cell lung cancer having distinctive biochemical, morphological, and growth properties. Cancer Res 45:2924–2930, 1985.
10. Kaye FJ, McBride OW, Battey J, Sausville EA: Human creatine kinase-B complementary DNA: Nucleotide sequence, gene expression in lung cancer, and chromosomal assignment to two distinct loci. J Clin Invest 79:1412–1420, 1987.
11. Sausville EA, Spindel E, Battey J, Sausville E: Expression of the gastrin-releasing peptide gene in human small cell lung cancer. Evidence for alternative processing resulting in three distinct mRNAs. J Biol Chem 261:2451–2457, 1986.
12. Said JW, Vimadala S, Nash G, Shinataky IP, Heusser RC, Sassoon AF, Lloyd RV: Immunoreactive neuron-specific enolase, bombesin, and chromogranin as markers for neuroendocrine lung tumors. Hum Pathol 16:236–240, 1985.
13. Bliss DP, Battey JF, Linnoila RI, Birrer MJ, Gazdar AF, Johnson BE: Expression of the atrial natriuretic factor gene in small cell lung cancer tumors and tumor cell lines. J Natl Cancer Inst 82:305–310, 1990.
14. Cuttitta F, Carney DN, Mulshine J, Moody TW, Minna JD: Bombesin-like peptides can function as autocrine growth factors in human small cell lung cancer. Nature 316:823–826, 1985.
15. Linoilla RI, Mulshine J, Steinberg S, Funa K, Matthews M, Cotelingham J, Gazdar A: Neuroendocrine differentiation in endocrine and nonendocrine lung carcinomas. Am J Clin Pathol 90:641–652, 1988.
16. Baylin SB, Gazdar AF: Endocrine biochemistry in the spectrum of human lung cancer: Implications for the cellular origin of small cell carcinoma. In: Greco F (ed): Small Cell Lung Cancer. New York, Grune and Stratton, 1981, pp 123–145.
17. Whang-Peng J, Bunn PA, Kao-Shan CS, Lee EC, Carney DN, Gazdar AF, Minna JD: A non-random chromosomal abnormality, del 3p(14–23). in human small cell lung cancer (SCLC). Cancer Genet Cytogenet 6:119–124, 1982.
18. Whang-Peng J, Kao-Shan CS, Lee EC, Bunn PA, Carney DN, Gazdar AF, Minna JD: Specific chromosome defect associated with human small cell lung cancer; deletion 3p(14–23). Science 215:181–182, 1982.
19. Brauch H, Johnson BE, Hovis J, Yano T, Gazdar A, Pettengill O, Graziano S, Sorenson G, Poiesz, Minna J, Linehan M, Zbar B: Molecular analysis of the short arm of chromosome 3 in small cell and non-small cell carcinoma of the lung. N Engl J Med 317:1109–1113, 1987.
20. Naylor SL, Johnson B, Minna J, Sakaguchi T: Loss of heterozygosity of chromosome 3p markers in small cell lung cancer. Nature 329:451–454, 1987.
21. Yokota J, Wada M, Shimosato Y, Terada M, Sugimura T: Loss of heterozygosity on chromosomes 3, 13, and 17 in small cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. Proc Natl Acad Sci USA 84:9252–9256, 1987.
22. Kok K, Osinga J, Carritt B, Davis MB, van der Hout AH, van der Veen AY, Landsvater RM, de Leij LF, Berendsen HH, Postmus PE, Poppema S, Buys CHC: Deletion of a DNA sequence at the chromosomal region 3p21 in all major types of lung cancer. Nature 330:578–581, 1987.
23. Johnson BE, Sakaguchi AY, Gazdar AF, Minna JD, Burch D, Marshall A, Naylor SL: Restriction fragment length polymorphism studies show consistent loss of chromosome 3p alleles in small cell lung cancer patients' tumors. J Clin Invest 82:502–507, 1988.
24. Drabkin H, Kao FT, Hartz J, Hart I, Gazdar A, Weinberger C, Evans R, Gerber M: Localization of human *erb*A-2 to the 3p22–3p24 region of chromosome 3 and variable deletion in small cell lung cancer. Proc Natl Acad Sci USA 85:9258–9262, 1988.
25. Leduc F, Brauch H, Hajj C, Dobrovic A, Kaye F, Gazdar A, Harbour J, Pettengill OS,

Sorenson GD, van der Berg A, Kok K, Campling B, Paquin F, Bradley WEC, Zbar B, Minna J, Buys C, Ayoub J: Loss of heterozygosity in a gene coding for a thyroid hormone receptor in lung cancers. Am J Hum Genet 44:282–287, 1989.
26. Mattei MG, de The H, Mattei JF, Marchio A, Tiollais P, DeJean A: Assignment of the human *hap* retinoic acid receptor RAR-beta gene to the p24 band of chromosome 3. Human Genet 80:189–190, 1988.
27. Gebert JF, Moghal N, Frangioni JV, Sugarbaker DJ, Neel BG: High frequency of retinoic acid receptor beta abnormalities in human lung cancer. Oncogene 6:1859–1868, 1991.
28. Harbour JW, Lai S-L, Gazdar AF, Minna JD, Kaye FJ: Expression in lung cancer of a transcribed sequence at the DNF15S2 locus at chromosome 3p21. Anticancer Res 10: 23–28, 1990.
29. Naylor SL, Marshall A, Hensel C, Martinez PF, Holley B, Sakaguchi AY: The DNF15S2 locus at 3p21 is transcribed in normal lung and small cell lung cancer. Genomics 4: 355–361, 1989.
30. LaForgia S, Morse B, Levy J, Barnea G, Cannizzaro LA, Li F, Nowell PC, Boghosian-Sell L, Glick J, Weston A, Harris CC, Drabkin H, Patterson D, Croce CM, Schlessinger J, Huebner K: Proc Natl Acad Sci USA 88:5036–5040, 1991.
31. Dunn JM, Phillips RA, Becker AJ, Gallie BL: Identification of germline and somatic mutations affecting the retinoblastoma gene. Science 241:1797–800, 1988.
32. Riordan JR, Rommens J, Kerem B, Alon N, Rozmahel R, Grzelczak J, Lok S, Plavsik N, Chou J, et al.: Identification of the cystic fibrosis gene: Cloning and characterization of complementary DNA. Science 245:1066–1073, 1989.
33. Cawthorne RM, Weiss R, Xu GF, Viskochil D, Culver M, Stevens J, Robertson M, Dunn D, Gesteland R, O'Connell P et al.: A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. Cell 62:193–201, 1990.
34. Sellers TA, Bailey-Wilson JE, Elston RC, Wilson AF, Elston GZ, Ooi WL, Rothschild H: Evidence for Mendelian inheritance in the pathogenesis of lung cancer. J Natl Cancer Inst 82:1272–1279, 1990.
35. Drabkin H, Bradley C, Patterson D: Translocation of c-*myc* in the hereditary renal cell carcinoma associated with a t(3;8) (p14.2;q24;13) chromosomal translocation. Proc Natl Acad Sci USA 82:6980–6984, 1985.
36. Teyssier J, Henry I, Dozier C, Ferre D, Adnet JJ, Pluot M: Recurrent deletion of the short arm of chromosome 3 in human renal cell carcinoma: Shift of the c-raf locus. J Natl Cancer Inst 77:1187–1191, 1986.
37. Zbar B, Brauch H, Talmadge C, Linehan M: Loss of alleles on the short arm of chromosome 3 in renal cell carcinoma. Nature 327:721–724, 1987.
38. Seizenger BR, Rouleou GA, Ozelius LJ, Lane AH, Farmer GE, Lamiell JM, Haines J, Yuen JWM, Collins D, Mahjoor-Krakauer D, Bonner T, Matthew C, Rubenstein A, Halperin J, McConkie-Rosell A, Green JS, Trofatter JA, Ponder BA, Eirman L, Bowmer MI, Schimke R, Oostro B, Aronin N, Smith DI, Drabkin H, Waziri MH, Hobbs WJ, Martuza RL, Conneally PM, Hsia YE, Gusella JF: Von Hippel-Lindau disease maps to the region of chromosome 3 associated with renal cell carcinoma. Nature 332:268–269, 1988.
39. Shimizu M, Yokota J, Mori N, Shuin T, Shinoda M, Terada M, Oshimura M: Introduction of normal chromosome 3p modulates the tumorigenicity of a human renal cell carcinoma cell line YCR. Oncogene 5:185–194, 1990.
40. Kao-Shan CS, Fine RL, Whang-Peng J, Lee EC, Chabner BA: Increased fragile sites and sister chromatid exchanges in bone marrow and peripheral blood of young cigarette smokers. Cancer Res 47:6278–6282, 1987.
41. Yokota J, Tsukada Y, Nakajima T, Gotoh M, Shimosato Y, Mori N, Tsunokawa Y, Sugimura T, Terada M: Loss of heterozygosity of the short arm of chromosome 3 in carcinoma of the uterine cervix. Cancer Res 49:3598–3601, 1989.
42. Ali IU, Lidereau R, Callahan R: Presence of two members of c-erbA receptor gene family (c-erbA beta and c-erbA2) in smallest region of somatic homozygosity on chromosome

3p21–p25 in human breast carcinoma. J Natl Cancer Inst 81:1815–1820, 1989.
43. Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM, Dryja TP: A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323:643–645, 1986.
44. Lee WH, Bookstein R, Hong F, Young LJ, Shew JY, Lee EY: Human retinoblastoma susceptibility gene: Cloning, identification, and sequence. Science 235:1394–1396, 1987.
45. Fung Y, Murphree A, T'Ang A, Qian J, Hinrichs SH, Benedict W: Structural evidence for the authenticity of the human retinoblastoma gene. Science 236:1657–1659, 1987.
46. Knudson AG, Jr.: Mutation and cancer: Statistical study of retinoblastoma. Proc Natl Acad Sci USA 68:820–823, 1971.
47. Yunis JJ, Ramsay N: Retinoblastoma and subband deletion of chromosome 13. Am J Dis Childh 132:161, 1978.
48. Cavanee WK, Dryja TP, Phillips RA, Benedict WF, Godbout R, Gallie BL, Murphree AL, Strong LC, White RL: Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. Nature 305:779–784, 1983.
49. Huang H, Yee JK, Shew JY, Chen PL, Bookstein R, Friedman T, Lee EY, Lee W-H: Suppression of the neoplastic phenotype by replacement of the *rb* gene in human cancer cells. Science 242:1563–1565, 1988.
50. Sumegi J, Uzvolgyi E, Klein G: Expression of the Rb gene under the control of MuLV-LTR suppresses tumorigenicity of WERI-Rb-27 retinoblastoma cells in immunodeficient mice. Cell Growth Differ 1:247–250, 1990.
51. Lee W-H, Shew J-Y, Hong FD, Sery TW, Donoso LA, Young L-J, Bookstein R, Lee EY-HP: The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. Nature 329:642–645, 1987.
52. Buchovich K, Duffy LA, Harlow E: The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell 58:1097–1105, 1989.
53. Chen P-L, Scully P, Shew J-Y, Wang JYJ, Lee WH: Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell 58:1193–1198, 1989.
54. DeCaprio JA, Ludlow JW, Lynch D, Furukawa Y, Griffin J, Piwinica-Worms H, Huang C-M, Livingston DM: The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell 58:1085–1095, 1989.
55. Ludlow JW, Shon J, Pipas JM, Livingston DM, DeCaprio JA: The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T. Cell 60:387–396, 1990.
56. Mihara K, Cao X-R, Yen A, Chandler S, Driscoll B, Murphree AL, T'Ang A, Fung Y-KT: Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. Science 246:1300–1303, 1989.
57. Xu H-J, Xu S-X, Hashimoto T, Takahashi R, Benedict WF: The retinoblastoma susceptibility gene product: A characteristic pattern in normal cells and abnormal expression in malignant cells. Oncogene 4:807–812, 1989.
58. Shenoy S, Choi J-K, Bagrodia S, Copeland TD, Maller JL, Shalloway D: Purified maturation promoting factor phosphorylates pp60c-src at the sites phosphorylated during fibroblast mitosis. Cell 57:763–774, 1989.
59. Lin BT, Gruenwald S, Morla AO, Lee W-H, Wang JY: Retinoblastoma cancer suppressor gene product is a substrate of the cell cycle regulator of cdc2 kinase. EMBO J 10:857–864, 1991.
60. Whyte P, Buchovich KJ, Horowitz JM, Friend SH, Raybuck M, Weinberg RA, Harlow E: Association between an oncogene and an antioncogene: The adenovirus E1A proteins bind to the retinoblastoma gene product. Nature 334:124–129, 1988.
61. DeCaprio JA, Ludlow JW, Figge J, Shew J, Huang C, Lee W, Marsilio E, Paucha E, Livingston DM: SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell 54:275–283, 1988.
62. Dyson N, Howley PM, Munger K, Harlow E: The human papilloma virus-16 E7

oncoprotein is able to bind to the retinoblastoma gene product. Science 243:934–937, 1989.
63. Kaelin WG, Pallas DC, DeCaprio JA, Kaye FJ, Livingston DM: Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. Cell 64:521–532, 1991.
64. Huang S, Lee W-H, Lee EY: A cellular protein that competes with SV40 T antigen for binding to the retinoblastoma gene product. Nature 350:160–162, 1991.
65. Chellappan SP, Hiebert S, Mudryj M, Horowitz JM, Nevins JR: The E2F transcription factor is a cellular target for the RB protein. Cell 65:1053–1061, 1991.
66. Rustgi AK, Dyson N, Bernards R: Amino-terminal domains of c-myc and N-myc proteins mediate binding to the retinoblastoma gene product. Nature 352:541–544, 1991.
67. Defeo-Jones D, Huang PS, Jones RE, Haskell KM, Vuocolo GA, Hanobik MG, Huber HE, Oliff A: Cloning of cDNAs for cellular proteins that bind to the retinoblastoma product. Nature 352:251–254, 1991.
68. Ludlow JW, DeCaprio JA, Huang C-M, Lee W-H, Paucha E, Livingston DM: SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. Cell 56:57–65, 1989.
69. Harbour JW, Lai S-L, Whang-Peng J, Gazdar AF, Minna JD, Kaye FJ: Abnormalities in structure and expression of the retinoblastoma gene in SCLC. Science 241:353–357, 1988.
70. Lee WH, Murphree AL, Benedict WF: Expression and amplification of the N-*myc* gene in primary retinoblastoma. Nature 309:458–460, 1984.
71. Little CD, Nau MM, Carney DN, Gazdar AF, Minna JD: Amplification and expression of the c-*myc* oncogene in human lung cancer cell lines. Nature 306:194–196, 1983.
72. Nau MM, Brooks BJ Jr., Carney DN, Gazdar AF, Battey JF, Sausville EA, Minna JD: Human small cell lung cancers show amplification and expression of the N-*myc* gene. Proc Natl Acad Sci USA 83:1092–1096, 1986.
73. Nau MM, Brooks BJ Jr., Battey J, Sausville E, Gazdar A, Kirsch I, McBride O, Bertness V, Hollis G, Minna J: L-*myc*: A new *myc*-related gene amplified and expressed in human small cell lung cancer. Nature 318:69–73, 1985.
74. Horowitz JM, Park S-H, Bogenmann E, Cheng J-C, Yandell DW, Kaye FJ, Minna JD, Dryja TP, Weinberg RA: Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells. Proc Natl Acad Sci USA 87:2775–2779, 1990.
75. Yokota J, Akiyama T, Fung Y-KT, Benedict WF, Namba Y, Hanaoka M, Wada M, Terasaki T, Shimosato Y, Sugimura T, Terada M: Altered expression of the retinoblastoma (RB) gene in small cell carcinoma of the lung. Oncogene 3:471–475, 1988.
76. Shew JY, Chen PL, Bookstein R, Lee EY, Lee WH: Deletion of a splice donor site ablates expression of the following exon and produces an unphosphorylated RB protein unable to bind SV40 T antigen. Cell Growth Differ 1:17–25, 1990.
77. Horowitz JM, Yandell DW, Park S-H, Canning S, Whyte P, Buchovich K, Harlow E, Weinberg RA, Dryja TP: Point mutational inactivation of the retinoblastoma antioncogene. Science 243:937–940, 1989.
78. Bookstein R, Shew J-Y, Chen P-L, Scully P, Lee W-H: Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated RB gene. Science 247:712–715, 1990.
79. Kratzke R, Shimizu E, Kaye FJ, unpublished.
80. Kaye FJ, Kratzke RA, Gerster JL, Horowitz JM: A single amino acid substitution results in a retinoblastoma protein defective in phosphorylation and oncoprotein binding. Proc Natl Acad Sci USA 87:6922–6926, 1990.
81. Hu Q, Dyson N, Harlow E: The region of the retinoblastoma protein needed for binding to adenovirus el A or SV40 large T antigen are common sites for mutations. EMBO J 9:1147–1155, 1990.
82. Huang H-JS, Wang N-P, Tseng BY, Lee W-H Lee EY-HP: Two distinct and frequently mutated regions of retinoblastoma protein are required for binding to SV40 T antigen. EMBO J 9:1815–1822, 1990.

83. Kaelin WG Jr., Ewen ME, Livingston DM: Definition of the minimal simian virus large T antigen- and adenovirus E1A-binding domain in the retinoblastoma gene product. Mol Cell Biol 10:3761–3769, 1990.
84. Hamel PA, Cohen BL, Sorce LM, Gallie BL, Phillips RA: Hyperphosphorylation of the retinoblastoma gene product is determined by domains outside the simian virus 40 large T antigen binding domain. Mol Cell Biol 10:6586–6595, 1990.
85. Reissmann PT, Koga H, Takahashi R, Benedict WF, Figlin R, Holmes EC, Slamon DJ, and the Lung Cancer Study Group: Inactivation of the retinoblastoma gene in non-small cell lung cancer. Proc Am Assoc Cancer Res 31:318, 1990.
86. Lee EY, To H, Shew JY, Bookstein R, Scully P, Lee WH: Inactivation of the retinoblastoma susceptibility gene in human breast cancer. Science 241:218–221, 1988.
87. Ginsberg AM, Raffeld M, Cossman J: Inactivation of the retinoblastoma gene in human lymphoid neoplasms. Blood 77:833–840, 1991.
88. Sanders BM, Jay M, Draper GJ, Roberts EM: Non-ocular cancer in relatives of retinoblastoma patients. Br J Cancer 60:358–365, 1989.
89. Takahashi R, Hashimoto T, Xu HJ, Hu SX, Matsui T, Miki T, Bigo-Marshall H, Aaronson SA, Benedict WF: Proc Natl Acad Sci USA 88:5257–5261, 1991.
90. Muncaster MM, Cohen BL, Phillips RA, Gallie BL: Failure of RB1 to reverse the malignant phenotype of human tumor cell lines. Cancer Res 1:654–661, 1992.
91. Cance WG, Brennan MF, Dudas ME, Huang CM, Cordon-Cardo C: Altered expression of the retinoblastoma gene product in human sarcomas. N Engl J Med 323:1457–1462, 1990.
92. Xu HJ, Hu SX, Cagle PT, Moore GE, Benedict WF: Absence of retinoblastoma protein expression in primary non-small cell lung carcinomas. Cancer Res 51:2735–2739, 1991.
93. Lane DP, Crawford LV: T antigen is bound to a host protein in SV40-transformed cells. Nature 278:261–263, 1979.
94. Linzer DIH, Levine AJ: Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal cells. Cell 17:43–52, 1979.
95. DeLeo A, Jay G, Appella E, Dubois G, Law L, Old L: Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. Proc Natl Acad Sci USA 76:2420–2424, 1979.
96. Jenkins JR, Rudge K, Currie GA: Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. Nature 312:651–654, 1984.
97. Parada LF, Land H, Weinberg RA, Wolf D, Rotter V: Cooperation between a gene encoding the p53 tumor antigen and ras in cellular transformation. Nature 312:649–651, 1984.
99. Eliyahu D, Raz A, Gruss P, Givol D, Oren M: Participation of p53 cellular tumor antigen in transformation of normal embryonic cells. Nature 312:646–649, 1984.
100. Hollstein M, Sidransky D, Vogelstein B, Harris CC: p53 mutations in human cancers. Science 253:49–53, 1991.
101. Finlay C, Hinds P, Levine AJ: The p53 proto-oncogene can act as a suppressor of transformation. Cell 57:1083–1093, 1989.
102. Eliyahu D, Michalovitz D, Eliyahu S, Pinhasi-Kimhi O, Oren M: Wildtype p53 can inhibit oncogene-mediated focus formation. Proc Natl Acad Sci USA 86:8763–8767, 1989.
103. Raycroft L, Wu HY, Lozano G: Transcriptional activation by wild-type but not transforming mutants of the p53 antioncogene. Science 249:1049–1051, 1990.
104. Fields S, Jang SK: Presence of a potent transcription activating sequence in the p53 protein. Science 249:1046–1049, 1990.
105. Donehower LA, Harvey M, Slagle BL, McArthur ML, Montgomery CA, Butel JS, Bradley A: Mice deificient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 356:215–221, 1992.
106. Malkin D, Li FP, Strong LC, Fraumeni JF Jr., Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA, Friend SH: Germline p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science 250:1233–1238, 1990.

107. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL: Genetic alterations during colorectal tumor development. N Engl J Med 319:525–532, 1988.
108. Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Preisinger AC, Jessup JM, van Tuinen DH, Barker DF, Nakamura Y, Vogelstein B: Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science 244:217–221, 1989.
109. Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P, Glover T, Collins FS, Weston A, Modali R, Harris CC, Vogelstein B: Mutations in the p53 gene occur in diverse human tumor types. Nature 342:705–708, 1989.
110. Takahashi T, Nau MM, Chiba I, Birrer M, Rosenberg R, Vinocur M, Levitt M, Pass H, Gazdar A, Minna J: p53: A frequent target for genetic abnormalities in lung cancer. Science 246:491–494, 1989.
111. Chiba I, Takahashi T, Nau MM, D'Amico D, Curiel DT, Mitsudomi T, Buchhagen DL, Carbone D, Piantadosi S, Koga H, Reissman PT, Slamon DJ, Holmes EC, Minna JD: Mutations in the p53 gene are frequent in primary, resected non-small cell lung cancer. Oncogene 5:1603–1610, 1990.
112. Iggo R, Gatter K, Bartek J, Lane D, Harris AL: Increased expression of mutant forms of p53 oncogene in primary lung cancer. Lancet 335:675–679, 1990.
113. Takahashi T, Takahashi T, Suzuki H, Hida T, Sekido Y, Ariyoshi Y, Ueda R: The p53 gene is very frequently mutated in small cell lung cancer with a distinct nucleotide substitution pattern. Oncogene 6:1775–1778, 1991.
114. Suzuki H, Takahashi T, Kuroishi T, Suyama M, Ariyoshi Y, Takahashi T, Ueda R: p53 mutations in non-small cell lung cancer in Japan: Association between mutations and smoking. Cancer Res 52:734–736, 1992.
115. Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 61:759–767, 1990.
116. Sidransky D, Frost P, Von Eschenbach A, Oyasu R, Presisinger AC, Vogelstein B: Clonal origin of bladder cancer. N Engl J Med 326:737–740, 1992.
117. Sidransky D, Mikkelsen T, Schwechheimer K, Rosenblum ML, Cavanee W, Vogelstein B: Clonal expansion of p53 mutant cells is associated with brain tumor progression. Nature 355:846–847, 1992.
118. Casson AG, Mukhopadhyay T, Leary KR, Ro JY, Levin B, Roth JA: p53 mutations in Barrett's epithelium and esophageal cancer. Cancer Res 51:4495–4499, 1991.
119. Puisieux A, Lim S, Groopman J, Ozturk M: Selective targeting of p53 gene mutational hotspots in human lung cancers by etiologically defined carcinogens. Cancer Res 51:6185–6189, 1991.
120. Vogelstein B, Kinzler KW: Carcinogens leave fingerprints [news]. Nature 355:209–210, 1992.
121. Morstyn G, Brown J, Novak U, Gardner J, Bishop J, Garson M: Heterogeneous cytogenetic abnormalities in small cell lung cancer cell lines. Cancer Res 47:3322–3327, 1987.
122. Miura I, Graziano S, Cheng J, Doyle A, Testa J: Chromosome alterations in human small cell lung cancer: Frequent involvement of 5q. Cancer Res 52:1322–1328, 1992.
123. Lukeis R, Irving L, Garson M, Hasthorpe S: Cytogenetics of non-small cell lung cancer: Analysis of consistent non-random abnormalities. Genes Chrom Cancer 2:116–124, 1990.
124. Whang-Peng J, Knutsen T, Gazdar A, Steinberg S, Oie H, Linnoila RI, Mulshine J, Nau M, Minna J: Nonrandom structural and numerical chromosome changes in non-small cell lung cancer. Genes Chromosomes Cancer 3:168–188, 1991.
125. Johnson BE, Battey J, Linoila RI, Becker KL, Makuch RW, Snider RH, Carney DN, Minna JD: Changes in the phenotype of human small cell lung cancer cell lines after transfection and expression of the c-myc proto-oncogene. J Clin Invest 78::525–532, 1986.
126. Wong A, Ruppert J, Eggleston J, Hamilton SR, Baylin SB, Vogelstein B: Gene

amplification of c-myc and N-myc in small cell carcinoma of the lung. Science 233:461–464, 1986.
127. Johnson BE, Ihde DC, Makuch RW, Gazdar A, Carney D, Oie H, Russell E, Nau M, Minna J: *myc* family oncogene amplification in tumor cell lines established from small cell lung cancer patients and its relationship to clinical status and course. J Clin Invest 79:1629–34, 1987.
128. Johnson BE, Makuch RW, Simmons AD, Gazdar AF, Burch D, Cashell AW: *myc* family DNA amplification in small cell lung cancer patients' tumors and corresponding cell lines. Cancer Res 48:5163–5166, 1988.
129. Brennan J, O'Connor T, Makuch RW, Simmons AW, Russell E, Linnoila RI, Phelps RM, Gazdar AF, Ihde DC, Johnson BE: *myc* family DNA amplification in 107 tumors and tumor cell lines from patient with small cell lung cancer treated with different combination chemotherapy regimens. Cancer Res 51:1708–1712, 1991.
130. Makela TP, Saksela K, Evan G, Alitalo K: A fusion protein formed by L-myc and a novel gene in SCLC, EMBO J 10:1331–1335, 1991.
131. Makela TP, Kere J, Winqvist R, Alitalo K: Intrachromosomal rearrangements fusing L-myc and rlf in small cell lung cancer Mol Cell Biol 11:4015–4021, 1991.
132. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM: Amplification of N-myc in untreated human neuroblastoma correlates with advanced disease state. Science 224:1121–1124, 1984, 1984.
133. Funa K, Steinholtz L, Nou E, Bergh J: Increased expression of N-myc in human small cell lung cancer biopsies predicts lack of response to chemotherapy and poor prognosis. Am J Clin Pathol 88:216–220, 1987.
134. Sklar MD: The ras oncogene increases the intrinsic resistance of NIH 3T3 cells to ionizing radiation. Science 237:645–647, 1988.
135. McKenna WG, Weiss MC, Endlich B, Ling CC, Bakanauskas VJ, Kelsten ML, Muschel RJ: Synergistic effect of the v-myc oncogene with H-ras on radioresistance. Cancer Res 50:97–102, 1990.
136. Rygaard K, Slebos RJ, Spang-Thomsen M: Radiosensitivity of small cell lung cancer xenografts compared with activity of c-myc, N-myc, c-raf-1, and K-ras proto-oncogenes. Int J Cancer 49:279–284, 1991.
137. Kaye FJ, Battey J, Nau M, Brooks B, Seifter E, DeGreve J, Birrer M, Sausville E, Minna J: Structure and expression of the human L-*myc* gene reveal a complex pattern of alternative mRNA processing. Mol Cell Biol 8:186–195, 1988.
138. Blackwood EM, Eisenman RN: Max: A helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with *Myc*. Science 251:1211–1217.
139. Blackwell TK, Kretzner L, Blackwood EM, Eisenman RN, Weintraub H: Sequence-specific DNA binding by the c-MYC protein. Science 250:1149–51, 1990.
140. Robbins PD, Horowitz JM, Mulligan RC: Negative regulation of human c-fos expression by the retinoblastoma gene product. Nature 346:668–671, 1990.
141. Pietenpol JA, Stein RW, Moran E, Yaciuk P, Schlegel R, Lyons RM, Pittelkow MR, Munger K, Howley PM, Moses HL: TGF-B1 inhibition of c-*myc* transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. Cell 61:777–85, 1990.
142. Zimmerman K, Yancopolous GD, Collum RG, Smith RK, Kohl NE, Denis KA, Nau MM, Witte O, Toran-Allerand D, Gee CE, Minna JD, Alt F: Differential expression of *myc* family genes during murine development. Nature 319:780–783, 1986.
143. Birrer MJ, Segal S, DeGreve JS, Kaye F, Sauseville EA, Minna JD: L-*myc* cooperates with *ras* to transform primary rat embryo fibroblasts. Mol Cell Biol 8:2668–2673, 1988.
144. Kawashima K, Shikama H, Imoto K, Izawa M, Naruke T, Okabayashi K, Nishimura S: Close correlation between restriction fragment length polymorphism of the L-myc gene and metastasis of human lung cancer to the lymph nodes and other organs. Proc Natl Acad Sci USA 85:2353–2356, 1988.
145. Tefre T, B:rrsesn AL, Aamdal S, Br:gger A: Studies of the L-myc DNA polymorphism

and relation to metastasis in Norwegian lung cancer patients. Br J Cancer 61:809–812, 1990.
146. Tamai S, Sugimura H, Caporaso NE, Resau JH, Trump BF, Weston A, Harris CC: Restriction fragment length polymorphism analysis of the L-myc gene locus in a case-control study of lung cancer. Int J Cancer 46:411–415, 1990.
147. Barbacid M: ras genes. Ann Rev Biochem 56:779–827, 1987.
148. Rodenhuis S, van de Wetering M, Mooi W, Evers S, van Zandwijk N, Bos J: Mutational activation of the K-*ras* oncogene: A possible pathogenetic factor in adenocarcinoma of the lung. N Engl J Med 317:929–935, 1987.
149. Rodenhuis S, Slebos RJC, Boot AJM, Evers S, Mooi W, Wagenaar S, Van Bodegom P, Bos J: K-*ras* oncogene activation in adenocarcinoma of the lung: Incidence and possible clinical significance. Cancer Res 48:5738–5741, 1988.
150. Suzuki Y, Orita M, Shiraishi M, Hayashi K, Sekiya T: Detection of ras gene mutations in human lung cancers by single-strand conformation polymorphism analysis of polymerase chain reaction products. Oncogene 5:1037–1043, 1990.
151. Mitsudomi T, Viallet J, Mulshine J, Linoila RI, Minna J: Mutations of ras genes distinguish a subset of non-small cell lung cancer cell lines from small cell lung cell lines. 6:1353–1362, 1991.
152. You H, Candrian U, Maronpot RR, Stoner GD, Anderson MW: Activation of the Ki-ras protooncogene in spontaneously occurring and chemically induced lung tumors of the strain A mouse. Proc Natl Acad Sci USA 86:3070–3074, 1989.
153. Quintinilla M, Brown K, Ramsden M, Balmain A: Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. Nature 322:78–80, 1986.
154. Slebos RJC, Kibbelaar RE, Daleiso O, Kooistra A, Stam J, Meijer CJLM, Wagenaar SS, Vanderschueren RG, van Zandwijk N, Mooi WJ, Bos JL, Rodenhuis S: K-ras activation as a prognostic marker in adenocarcinoma of the lung. N Engl J Med 323:561–565, 1990.
155. Mitsudomi T, Steinberg S, Oie H, Mulshine J, Phelps R, Viallet J, Pass H, Minna J, Gazdar A: ras gene mutations in non-small cell lung cancers are associateed with shortened survival irrespective of treatment intent. Cancer Res 51:4999–5002, 1991.
156. Cline M, Battifora H: Abnormalities of proto-oncogenes in non-small cell lung cancer. Correlation with tumor types and clinical characteristics. Cancer 60:2669–2674, 1987.
157. Griffin CA, Baylin SB: Expression of c-*myb* oncogene in human small cell lung cancer. Cancer Res 45:272–275, 1985.
158. Kiefer PE, Bepler G, Kubasch M, Havemann K: Amplification and expression of proto-oncogenes in human small cell lung cancer cell lines. Cancer Res 47:6236–6242, 1987.
159. Sekido Y, Obata Y, Ueda R, Hida T, Suyama M, Shimokata K, Ariyoshi Y, Takahashi T: Preferential expression of c-kit protooncogene transcripts in small cell lung cancer. Cancer Res 51:2416–2419, 1991.
160. Kaye F: Lung cancer. In: Cossman J (ed): Molecular Genetics in Cancer Diagnosis. Elsevier Science, New York, 1990, pp 431–442.
161. Rosenfeld MA, Siegfried W, Yoshimura K, Yoneyama K, Fukayama M, Stier LE, Paakko PK, Gilardi P, Stratford-Perricaudet LD, Perricaudet M, Jallet S, Pavirani A, Lecocq J-P, Crystal RG: Adenovirus-mediated transfer of a recombinant alpha-1-antitrypsin gene to the lung epithelium *in vivo*. Science 252:431–434, 1991.
162. Rosenfeld MA, Yoshimura K, Trapnell B, Yoneyama K, Rosenthal ER, Dalemans W, Fukuyama M, Bargon J, Stier LE, Stratford-Perricaudet L, Perricaudet M, Guggino WB, Pavirani A, Lecocq J-P, Crystal RG: In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. Cell 68:143–155, 1992.

4. Thyroid growth factors and oncogenes

Orlo H. Clark and Quan-Yang Duh

Introduction

Thyroid neoplasms and thyroid enlargement result from the preferential growth (benign), invasion, and/or metastases (malignant) of either follicular or parafollicular cells in the thyroid gland. By histological examination at autopsy and by sensitive localization procedures such as ultrasound, about 50% of persons have thyroid tumors [1,2]. Although most thyroid tumors are benign, thyroid cancer has been documented at postmortem examinations in 13% of person in Minnesota and up to 28% of Japanese in Hawaii [3,4]. Most of these tumors are occult and of little clinical consequence. Clinically appreciable enlarged thyroid glands, however, occur in about 4% of the population in the United States and are more common in areas of endemic goiter [5]. Diffuse and nodular hyperplasia of the thyroid gland with or without hyperfunction (hyperthyroidism) is also a relatively common clinical problem.

Thyroid cancer requiring clinical treatment occurs in about 40 persons per million population per year and 6 persons per million die of thyroid cancer annually in the United States [6]. Thyroid cancers vary dramatically in behavior; small (less than 1 cm) occult sclerosing papillary cancers are rarely lethal, whereas most patients with anaplastic thyroid cancer die within 6 months of diagnosis, despite all means of therapy [7]. Follicular thyroid cancer and anaplastic thyroid cancer and death due to thyroid cancer all occur more commonly in areas of endemic goiter and iodine deficiency [8,9]. Papillary thyroid cancer is more common in areas of iodine excess [10,11]. Thus the follicular cell may develop into different tumors based on iodine consumption. The various types of thyroid malignancies are listed in Table 1.

Other factors also appear to predispose one to developing thyroid cancer, including heredity [12,13], radiation exposure [14], carcinogens [15], and to a lesser degree estrogens [16], antecedent breast cancer [17], dietary calcium and vitamin D [18], and living near certain types of volcanoes [19]. A recent study suggests that patients with benign thyroid disorders are more prone to develop malignant thyroid disorders, suggesting either common environ-

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Table 1. Type of thyroid malignancies

Follicular cell origin	Stroma
Papillary	Lymphoma
Follicular	Sarcoma
Mixed	Teratoma
Hurthle cell	
Anaplastic	
Parafollicular cell origin	
Medullary	
Anaplastic	

mental factors or that benign tumors such as follicular adenomas may progress to follicular adenocarcinomas [20]. The presence of Hashimoto's thyroiditis predisposes patients to develop thyroid lymphoma [21]. The association of various epidemiologic factors and thyroid cancer, including genetic predisposition and the environment, provide an opportunity to study these factors and their relation to tumorigenesis.

Thyroid tumors, because of their marked variation in aggressiveness, their relation to predisposing factors, their response to thyroid stimulating hormone (TSH), and other endocrine, paracrine, and autocrine growth factors offer an ideal system to study the role of oncogenes and tumor suppressor genes, as well as changes in signal transduction pathways and their relationship to the development and progression of these neoplasms. TSH has been documented to stimulate the adenylate cyclase-protein kinase A pathway, as well as the phospholipase C-protein kinase C pathway [21–24].

Thyroid growth factors

In areas of endemic goiter (iodine deficiency) up to 85% of the population have goiters [25]. Even animals in these areas have goiters [26]. Iodine deficiency increases the thyroid growth response to TSH [27], and also, by decreasing thyroid hormone levels, secondarily increases serum TSH levels that stimulate thyroid growth. When iodine intake is increased in these areas, the frequencies of both nodular goiter and thyroid cancer decrease [28]. A recent investigation documented that iodine deficiency decreased the concentration of transforming growth factor (TGF) beta, a growth factor that inhibits thyroid epithelial growth [29]. Transforming growth factors are substances that tumors secrete (autocrine factors) that allow tumors to grow in the absence of serum or other growth factors. It is now recognized that TGF-alpha and -beta are also involved in normal cell growth regulation. Increased consumption of vitamin A has been reported to result in smaller thyroid gland size [30]. In contrast, a high-calcium diet, especially in areas of

Table 2. Endocrine, paracrine and autocrine factors as thyroid growth factors

Stimulators	Inhibitors
Thyroid stimulating hormone (TSH)	Transforming growth factor beta (TGF-β)
Thyroid stimulating immunoglobulins (TSI)	Vitamin A
Epidermal growth factor (EGF)	
Insulinlike growth factor 1 & 2 (IGF)	
Fibroblast growth factor (FGF)	
Human chorionic gonadotropin (HCG)	
Growth hormone (GH)	
Iodine deficiency	
Interleukin-1 (IL-1)	
Platelet-derived growth factor (PDGF)[a]	
Prostaglandin E_2 (PGE_2)	
Vasoactive intestinal polypeptide (VIP)	
Vitamin C	

[a] Anaplastic thyroid cancer only.

Table 3. Oncogenes as thyroid growth factors

Circulating growth factors	*sis* (PDGF)
	TSI, TSH[a]
	int-2 (FGF)
Plasma membrane receptors	c-*erb*B (EGF receptor)
	PTC
	neu (EGF-receptor)
Signal transduction	*ras* (p21 proteins)
	gsp (stimulating G protein)
Nuclear transcription factors	*myc*
	fos
	jun

[a] TSI, TSH, and TSH receptors are involved in the growth regulation of thyroid cells but not other cells, and may be considered tissue-specific 'oncogenes.'

relative iodine deficiency, results in goiter (Derbyshire neck) [31]. Goiter is also more common in parts of West Virginia and in other areas where coal or oil production is common, even when there is no iodine deficiency [32].

The list of thyroid growth factors and inhibitors is found in Table 2. These factors often work in concert, and the relative importance of individual growth factors may vary in different species. Experts currently agree that one can no longer assume that there is one hormone, such as thyroid stimulating hormone (TSH), affecting one target tissue — the thyroid [33]. For example, estrogen has little trophic effect on breast cells in culture unless other growth factors, such as serum or fibrolasts, are present in the culture media [34]. Endocrine growth factors for the thyroid gland include TSH, thyroid stimulating immunoglobulins (TSI), and human chorionic

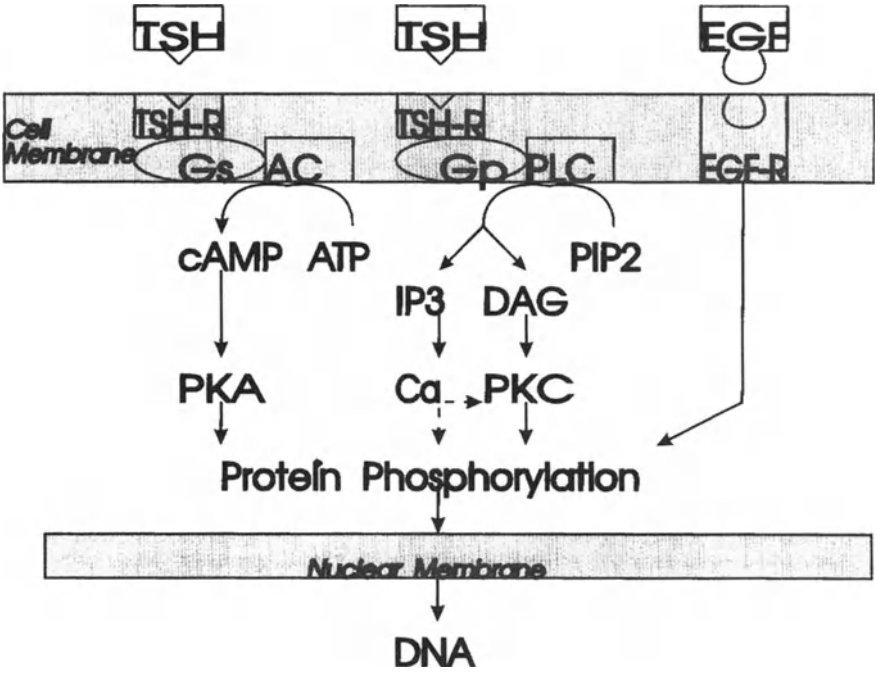

Figure 1. TSH-adenylate cyclase-protein kinase A, TSH-phospholipase C-protein kinase C, and EGF-tyrosine kinase signal transduction pathways in a thyroid cell.

gonadotropins (HCG). Pleiotropic auto or paracrine growth factors that act to regulate thyroid cell growth include epidermal growth factor (EGF), fibroblast growth factors (FGF), insulinlike growth factors (IGF), platelet-derived growth factor (PDGF), interleukin 1, transferrin, prostaglandin, and other stimulating growth factors as well as factors that appear to inhibit growth, such as transforming growth factor beta, iodine, somatostatin, adenosine, lithium, alpha-adrenergic agents, and vitamin A [23,35]. Thus both extracellular and intracellular growth factors or inhibitors act on their receptors to regulate thyroid growth.

To understand how various growth factors function, one must understand the signal transduction pathways that occur within a cell. In the thyroid gland TSH, TSI, EGF, IG-1, IGF-2, FGF, vasoactive intestinal polypeptide (VIP), and interleukin (IL) have been described to have growth effects [23,24,35,36]. Some of these growth factors, such as TSH, TSI, and VIP, work via the adenylate-cyclase protein kinase A system, others work via the phosphoinositide-protein kinase C system, and others work via tyrosine kinase (Fig. 1). Currently we do not know how an endocrine cell knows when to secrete a hormone and when to grow in response to a specific stimulus. For example, TSH and TSI appear to stimulate growth and dif-

ferentiated function, such as iodine incorporation in normal thyroid tissue, whereas EGF increases growth but decreases differentiated function [37].

It appears that for each step in the hormone or growth factor-receptor pathway, there are both positive and negative regulating steps. These regulating steps are controlled by certain genes and their products. Thus, cell proliferation is regulated by growth-promoting proto-oncogenes, counterbalanced by growth-constraining tumor suppressor genes. Mutations that potentiate the activities of proto-oncogenes create oncogenes (genes that are either overproduced or whose mutation results in an unregulated protein product) that influence growth. Oncogenes and tumor suppressor genes or their products can mimic or replace specific growth factors, growth factor receptors, signal transducers, or nuclear transcription factors as shown in Table 3 [38–41].

Oncogenes and basic molecular biology

Oncogenes are usually less responsive to the regulatory mechanisms that control normal cell growth than proto-oncogenes [42–44]. The genetic alterations that change proto-oncogenes to oncogenes provide a cell with a growth advantage and are usually dominant mutations [45]. Oncogenes are created from proto-oncogenes via point mutations, inversions, translocations, or amplifications.

Genes are coded by double-stranded linear deoxyribonucleic (DNA) molecules. Four different bases of DNA — adenine (A), guanine (G), thymidine (T), and cytosine (C) — make up this code. Genes include coding sequences of DNA (exon), spacer regions (introns), and regulatory sequences (promotors/enhancers) that direct the expression of adjacent or perhaps other protein-coding regions by attracting protein-coding regions. Three bases in specific order — the codon — encode for a particular amino acid, e.g., ATG for methionine. Mutations are changes in DNA base sequences that may result from (1) change in a singe base or base pair, termed a *point mutation*; (2) loss of a variable length of DNA, ranging from one base to entire genes to whole chromosomes (deletion); or (3) *rearrangements* between genetic loci on the same (inversion) or different (translocations) chromosomes. A tumor can contain several different chromosomal abnormalities.

Mutations may be inherited and either result in death of the cell or fetus or may predispose one to familial cancers. Other mutations may be acquired, such as DNA damage resulting from radiation or carcinogens. Thyroid tumors occur in up to 50% of children exposed to low-dose (6.5–2000 rads) radiation and about 7% of such children develop thyroid cancer [46]. Carcinogens also cause DNA damage and thyroid tumors [15].

DNA codes a single-stranded base sequence called ribonucleic acid (RNA) [47,48]. RNA exists in four forms: transfer RNA, ribosomal RNA, small nuclear ribonucleoprotein particle (SNRP), and messenger RNA

(mRNA). The mRNA carries the coded sequence of DNA from the nucleus to the cytoplasm for translation into proteins. Proteins are the major functional molecules in the cell. The amino acids in a protein determine its shape, function, and activity.

Both DNA and RNA viruses can cause tumors and are called viral oncogenes. These viral oncogenes encode for proteins that appear to be involved in the growth of some thyroid neoplasms. Oncogenes have been documented for growth factors (*sis*), growth factor receptors (*erb*B), signal transducers (*ras*, *gsp*), and regulation of gene transcription (*fos*, *jun*, *myc*) [42,45,49–56]. Thus, after infection of cells the viral DNA is integrated into the host genome. Such infection may result in cellular transformation via the chronic production of one or more proteins. This results in enhanced DNA synthesis [50,57] and can transform various cells in vivo or in culture so that they no longer require the usual growth factor. The introduction of gene coding for simian virus (SV40T) antigen into transgenic mice, for example, results in thyroid and other endocrine tumors [58]. Thus, the overexpression of normal genes, expression of new or altered genes, and the deletion of suppressor genes can all result in cellular transformation and malignant tumors. Viral oncogenes can cause these types of cellular transformations.

A loss of genetic material may also cause tumors by the absence of a growth regulator that normally constrains cellular proliferation. Loss of these tumor suppressor genes (recessive oncogenes or anti-oncogenes) usually requires that both alleles are deleted. The best example of a gene that encodes products that suppress the development of malignancy is the tumor retinoblastoma gene [59]. Patients with familial retinoblastoma have a dominantly inherited mutation in the retinoblastoma gene that is situated in one of the two alleles of chromosome 13. Patients who acquire a second mutation in the previously normal gene on the other allele of chromsome 13 develop retinoblastoma because this mutation eliminates the cell's ability to produce enough of the tumor suppressor gene product to prevent tumor development. This type of anti-oncogene may also be involved in patients who develop familial thyroid cancers [60].

The chromosomal abnormalities responsible for the dominantly inherited multiple endocrine neoplasm (MEN) types 1, 2a, and 2b have been identified. The gene responsible for MEN 1 has been mapped to chromosome 11, and the gene for MEN 2 has been mapped to chromosome 10 [61–64]. The MEN syndrome involves multiple tumors and/or hyperplastic changes in the parathyroid glands, pituitary, and pancreas. Patients with MEN 2a have tumors of the thyroid (medullary), and the adrenal (pheochromocytoma) and parathyroid glands. Patients with MEN 2b have tumors of the thyroid and adrenal but rarely of the parathyroid [65]. The latter patients also have a characteristic Marfanoid habitus with mucosal neuromas and ganglioneuromas of the gastrointestinal tract [65]. Medullary thyroid cancers are more lethal in patients with MEN 2b than in MEN 2a or sporadic medullary thyroid cancer MTC [66,67]. Patients with familial medullary thyroid cancer with-

out other manifestations of MEN 2 have the best prognosis, and in some families patients die with medullary thyroid cancer but not from medullary carcinoma. It, therefore, appears that identification of the specific chromosomal abnormality in patients with medullary thyroid cancer will not only be valuable in screening family members for the predisposition to medullary thyroid cancer but also for predicting the biological behavior of this tumor.

TSH

There are considerable clinical and laboratory evidence supporting TSH as a trophic hormone for the thyroid gland and for thyroid neoplasms. Bruns in 1895 [68], in 326 patients with nodular goiters, reported that about two thirds of the goiters decreased in size in response to treatment with thyroid hormone (presumably by decreasing serum TSH levels). Subsequent studies by Greer and Astwood [69], Shimoaka and Sokel [70], and others [71,72] confirmed these studies, but more recent studies by Boey et al. [73] and Morita et al. [74] documented that only about 40% of thyroid nodules decreased in size. A well-designed investigation by Gharib et al. [75], however, found that no significant regression in thyroid nodules occurred in patients treated with thyroid hormone when compared to placebo-treated control patients. Berghout et al. [76], however, recently documented the effectiveness of treating patients with nodular goiter with thyroid hormone when compared to placebo-treated controls and also noted increased goiter growth when the thyroid hormone was discontinued (Fig. 2).

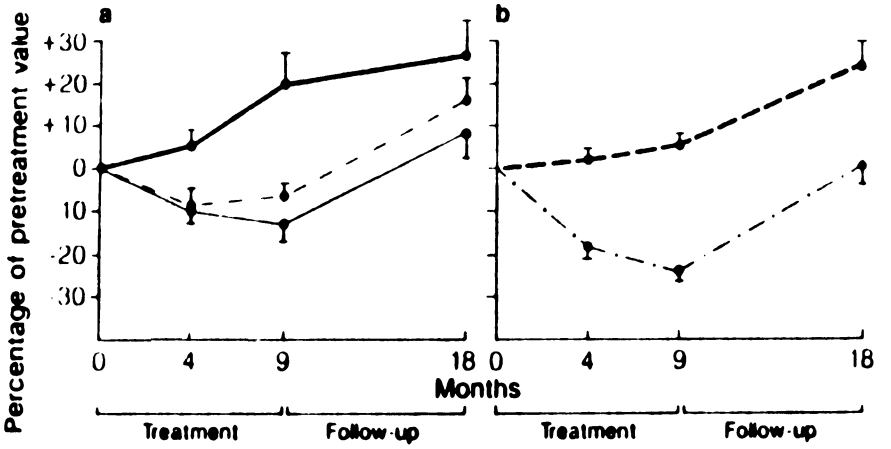

Figure 2. Effect of TSH suppressors with L-thyroxine (2.5 mg/thyroxin kg body unit daily) alone or combined with carbinazole (CBZ, 40 mg daily) in 78 patients with sporadic nontoxic goiter in a prospective placebo-controlled double-blind randomized trial. A response to treatment as judged by ultrasonography was found in 58% of the T4-treated group, in 35% of the T4-carbinazole-treated group, and in 5% of the placebo-treated group. From Berghout et al. [76], with permission.

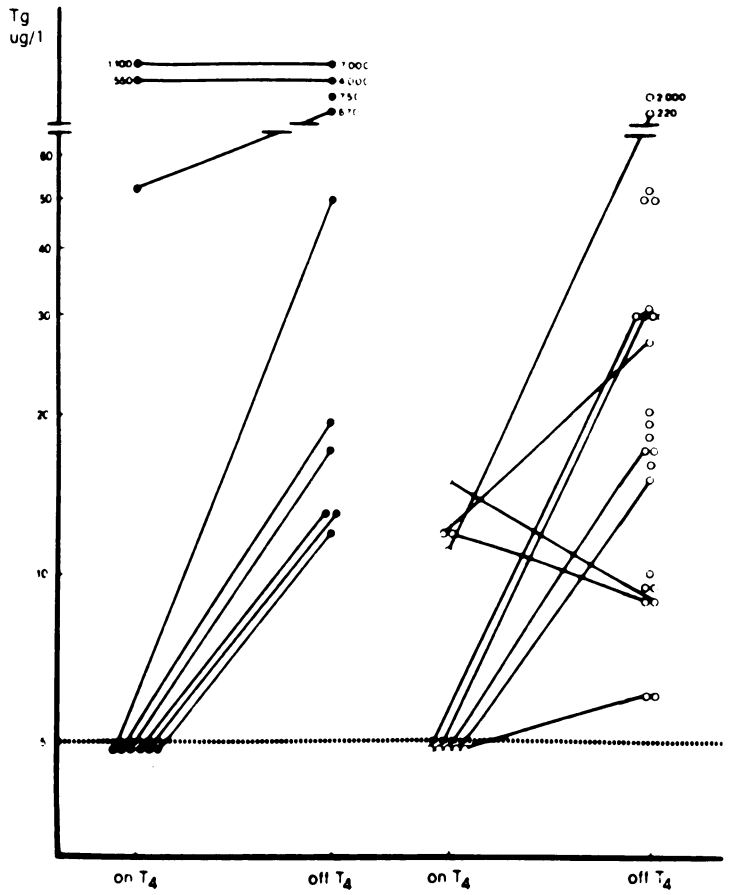

Figure 3. Serum thyroglobulin levels after thyroidenctomy in patients whose metastatic thyroid cancers did (solid circles) or did not (open circles) take up radioactive iodine. From Grant et al. [87], with permission.

Dunhill [77] in 1934 was the first to report that some thyroid carcinomas regress in response to treatment with thyroid hormone. His clinical observation was confirmed by Crile [71] and Thomas [72], and Balme [78] even reported regression of metastatic thyroid cancer. Purves et al. [79] documented that rats fed goitrogens develop thyroid tumors, and numerous investigators have demonstrated that treatment with thyroid hormone prevents or decreases the number of radiation-induced thyroid cancers in rats [80–82]. Fogelfeld et al. [83,84] reported that in people exposed to low-dose therapeutic radiation thyroid hormone decreased tumor recurrence. Studies of DeGroot et al. [85], in contrast, failed to find any beneficial effects.

High serum TSH levels are also required for effective transplantation of most thyroid tumors [86]. Increased TSH levels in patients with persistent papillary and follicular thyroid cancer, even after total thyroidectomy,

increase serum thyroglobulin levels, thus demonstrating the importance of suppressing serum TSH levels [87]. (Fig. 3). Serum thyroglobulin levels decrease in most patients with thyroid nodules whose nodules decrease in size in response to TSH suppression therapy [74]. Numerous studies have also documented that most differentiated thyroid tumors of follicular cell origin have TSH receptors, have an intact TSH-adenylate cyclase signal transduction system, and respond metabolically to TSH [33,88,89]. Both functioning and nonfunctioning thyroid tissue transplanted into nude mice also appears not only to grow faster than normal human thyroid tissue in response to TSH stimulation, but also suppression of TSH decreases this growth [90]. The amount of regression in growth of abnormal thyroid tissue, however, is not as great as that which occurs in normal thyroid tissue, suggesting that other thyroid growth factors are also involved.

Thyroid stimulating immunoglobulins (TSI) or antibodies also stimulate normal thyroid tissue and cause goiter (Graves' disease) [91,92]. These immunoglobulins may also stimulate the growth of some thyroid tumors [93]. TSI appears to function via the TSH receptor. In one study TSI stimulated the adenylate cyclase system but not the phosphoinositide phospholipase C system (Fig. 1) [94]. The cytokines, such as interleukin, are also thyroid growth factors, and the local release of these substances by lymphocytes and other cells within the thyroid gland or thyroid tumor could influence normal or neoplastic thyroid growth [95–97].

Thyroid oncogenes

Oncogenes, whether of viral origin or not, encode products that act either in the nucleus or in the cytoplasm [42]. Specific oncogenes have been documented in thyroid neoplasms. These oncogenes often cause constitutive activation of the signal transduction pathway. For example, *sis* and *int*-2 oncogenes resemble platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), respectively [49,50,98–100]. The *sis* oncogene and PDGF receptors that are not usually found in normal follicular cells have been identified in two anaplastic thyroid cancer cell lines and FGF has been reported to increase thyroid growth [98–100]. The ras oncogene product or p21 is a guanyl nucleotide binding protein that keeps GTP bound in an 'activated state.' The stimulating G protein oncogene (*gsp*) has been found in thyroid neoplasms, whereas the inhibiting G-protein oncogene (*gip*) has not [52]. TSH stimulation increases c-*myc* and c-*fos* oncogene expression in cultured thyroid cells [49,55]. Terrier et al. [101] documented increased amounts of c-*myc* RNA in 57% (13 of 23) of the thyroid cancers they studied and increases in c-*fos* RNA in 61% (14/23). Patients with an unfavorable clinical and/or histological programs were twice as likely to overexpress c-*myc* as patients with a better prognosis. The expression of c-*fos* did not correlate with prognosis.

The studies by Lemoine et al. [51,102–110] have documented that *ras* is present in 0–100% of thyroid neoplasms. The three types of activated *ras* oncogenes (N-*ras*, H-*ras*, and K-*ras*) vary in thyroid neoplasms. Wright et al. [111] recently reported that although the overall prevalence of *ras* mutations was the same in 68 spontaneous and 12 radiation-associated thyroid tumors, follicular carcinomas that developed in radiated patients had a significantly higher K-*ras* mutation rate (60–67% $p < 0.005$). Johnson et al. [112] noted that expression of the *ras* oncogene product, p21, is only weakly expressed in normal thyroid tissue but is more strongly expressed in both benign and malignant thyroid tumors, as well as in Hashimoto's thyroiditis and Graves' disease. Anaplastic carcinoma, however, showed little staining for p21. Pacini et al. [113] documented that p21 staining was positive in 35 of 51 (64.8%) thyroid tumors. A significant correlation was found between patient outcome and p21 tumor staining. Eleven of 12 patients who died (91.6%) with papillary thyroid cancer and 8 of 17 (72.7%) patients with follicular cancer had tumors that stained positive for p21. Overall 19 of 23 (82.6%) of patients who died stained positively for p21 vs. a 51.5% positive rate in patients who were alive ($p < 0.02$ by Chi square). This is in contrast to those findings of Nakagawa et al. [114], who documented that introduction of v-H-*ras* oncogene into an aggressive human medullary thyroid cancer cell line that did not normally express H-*ras* induced differentiation and a decrease in cellular proliferation. PC12, a phenochromocytoma cell line, also became more differentiated in response to microinjection of *ras*-p21 [115]. Thus the same oncogenes may manifest in different ways in varying tumors.

Shi et al. [116] reported that mutated ras oncogene is more common in thyroid tumors from iodine-deficient patients from Hungary than in iodine-sufficient patients from Newfoundland. We, however, have not observed these differences in *ras* oncogenes in thyroid tumors from iodine-deficient Germany when compared to patients from iodine-rich San Francisco [117]. Iodine deficiency could be a reason for some differences in *ras* mutations observed in various studies. Thus, Lemoine et al. [102–104] report a higher frequency of *ras* mutation in follicular thyroid cancer in patients than do most other groups, and these investigators work in a relatively low iodine area. Other factors may also be important for the reported discrepancies in mutant *ras* frequency in thyroid tumors. These include (1) varying techniques with conditions not stringent enough to distinguish mutant from wild-type DNA sequences in some investigations, (2) tumor selection and classification, and (3) contamination by stromal or normal thyroid. Genetic predisposition and environmental exposure to radiation and carcinogens or infectious agents that serve as mutagens might also contribute to the observed differences. For example, Lidereau et al. [118] have observed the association of H-*ras* and tumor susceptibility, especially the development of breast cancer. Muschel et al. [119] noted in NIH-3T3 cells that activated

H-*ras* and another as of yet undefined factor are required for the development of metastatic disease.

Lyons et al. [52] have reported a GTPase inhibiting mutation in the alpha chain of the heterotrimeric G protein. This mutation constitutively activates the G protein and is called the *gsp* oncogene. In initial studies, this oncogene was present in 14 of 42 (43%) growth hormone secreting pituitary tumors but in only one of 24 (4%) thyroid tumors. One would predict that *gsp* oncogenes would result in constitutive activation of adenylate cyclase, since they inhibit the guanosine triphosphatase (GTPase) activity. This proved to be true for the GH-secreting pituitary tumors that have a *gsp* mutation but not for the single *gsp*-positive thyroid autonomous follicular adenoma. Suarez et al. [53] recently reported that 2 of 3 constitutively activated follicular carcinomas and 1 of 3 constitutively activated papillary thyroid carcinomas had a *gsp* oncogene. This suggests that the *gsp* oncogene may be an important oncogene, but it also suggests that other factors result in constitutive activation of the adenylate cyclase pathway, since 3 of the 6 patients were *gsp* negative. O'Sullivan et al. [120] identified *gsp*-activating mutations in 5 of 13 (38%) autonomously functioning thyroid adenomas, but in 0 of 16 nonfunctioning adenomas, six papillary carcinomas, and three follicular carcinomas. They suggest that the *gsp* oncogene is involved in the pathogenesis of autonomously functioning thyroid tumors but not in other tumors. Studies by Goretzki et al. [121] have documented that when both *gsp* and *ras* mutations are present in thyroid cancer, the tumor behaves in a more aggressive fashion. The *gsp* oncogene, i.e., activating mutations of the Gs gene, have been found in the various tissues from four patients with the McCune-Albright syndrome, which is characterized by polyostatic fibrous dysplasia, sexual precosity, and hyperfunction of various endocrine glands, including multinodular goiter [122].

Fusco et al. [123] have reported the presence of a new oncogene named *PTC* in papillary thyroid cancers, as well as in two lymph node metastases. This gene is a variant of the *ret* proto-oncogene. *ret* proto-oncogenes were also identified in human pheochromocytomas and in medullary thyroid carcinoma of the sporadic and familial type [124]. The latter finding suggested that the *ret* gene would be on situated on chromosome 10, close to the gene that predisposes patients to the MEN 2a syndrome. Donghi et al. [125] subsequently found the *PTC* oncogene on chromosome 10 q11-q12 in the same region as that for multiple endocrine neoplasia type 2a (MEN 2a). The same investigators screened 20 papillary thyroid cancers from 16 patients. DNA from thyroid tumors from 10 patients (62%) displayed a transforming activity in cultured cells: 4 due to the *PTC* oncogene, 4 due to *TRK* oncogene, and two due to N-*ras*. Since both *PTC* and *TRK* have tyrosine kinase activity and the EGF receptor is also a tyrosine kinase, this signal transduction pathway may also be involved in the pathogenesis of papillary thyroid cancer [126].

The p53 gene acts as a tumor suppressor gene or as a dominant transforming oncogene (see chapter 17). Wild-type p53 has been documented to be antiproliferative in SV40-transformed HR 8 cells, colon carcinoma cells, and other cells [60,127]. Mutant p53 genes are found in breast, colon, and lung cancers. Only 1 of 130 thyroid tumors, however, was found to have a p53 mutation [127]. Recently, however, p53 mutations were detected in 6 of 7 undifferentiated thyroid carcinomas [128].

Conclusions

The marked variations in thyroid tumor aggressiveness, the known regulatory control by thyroid stimulating hormone (TSH), and other growth factors makes thyroid tumors an ideal system to study. The TSH-adenylate cyclase signal transduction pathway has been extensively studied in thyroid neoplasms and information is also available about TSH-phospholipase C, EGF-tyrosine kinase, and other signaling systems. Numerous oncogenes have also been found in both benign and malignant thyroid tumors, including *ras*, *gsp*, *erb*B, *sis*, *ret*, *TRK*, *PTC*, *myc*, *fos*, and *jun*. Several studies, but not all, suggest that the presence of several oncogenes predicts a more aggressive tumor behavior.

It appears that more aggressive thyroid cancers may contain more activated oncogenes and/or inactivated anti-oncogenes, similar to what has been found in the studies of colon cancer [129]. Analyzing the oncogene profile of a tumor may help in the diagnosis of certain tumors, in predicting the behavior of tumors, and thus how aggressive patients with these tumors should be treated. Recent studies have documented that removal of a single genetic lesion eliminates the ability of cancer cells to grow in nude mice [130]. For such therapy to be applicable for patients, methods must be developed to deliver gene constructs to the cancer cells, possibly via viral vectors. Thus the expression of a dominant oncogene could be inhibited, or a deleted or altered tumor suppressor gene could be replaced. Techniques may also be developed with oncogene-specific monclonal antibodies or other substances that can interfere with the excessive stimulus for growth or metastases.

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References

1. Mortenson JD, Wooler LB, Bennett WA: Gross and microscopic findings in clinically normal thyroid glands. J Clin Endocrinol Metab 15:1270–1280, 1955.

2. Stark DS, Clark OH, Gooding GAW, Moss AA: High-resolution ultrasonography and computed tomography of thyroid lesions in patients with hyperparathyroidism. Surgery 94:863–868, 1983.
3. Nishiyama RH, Ludwig GK, Thompson NW: The prevalence of small papillary thyroid carcinomas in 100 consecutive necropsies in an American population. In: Nishiyama RH, Ludwig GK, Thompson NW, De Groot LJ (eds): Associated Thyroid Carcinoma. Grune & Stratton, New York, 1976, pp 123–125.
4. Fukunaga FT, Yatani R: Geographic pathology of occult thyroid carcinomas. Cancer 36:1095–1099, 1975.
5. Greenspan FS: Medical treatment of nodular goiters. In: Greenspan FS, Clark OH (eds): Endocrine Surgery of the Thyroid and Parathyroid Glands. CV Mosby, St. Louis, 1985, pp 35–55.
6. Culter SJ, Young JL (eds): Third National Cancer Survey incidence data. National Cancer Institute. Monograph No. 41. Department of Health Education and Welfare (NIH), Washington DC, 1975.
7. Clark OH: Thyroid nodules and thyroid cancer. In: Clark OH, Clark OH (eds): Endocrine Surgery of the Thyroid and Parathyroid Glands. CV Mosby, St. Louis, 1985, pp 56–90.
8. Wahner HW, Cuello C, Correa P, Uribe LF, Gaitan E: Thyroid carcinoma in an endemic goiter area, Cali, Columbia. Am J Med 40:58–66, 1966.
9. Vigneri R: Studies on the goiter endemia in Sicily. J Endocrinol Invest 11:831–843, 1988.
10. Beierwaltes WH: Iodine and lymphocytic thyroiditis. Bull All India Ins Med Sci 3: 145–151, 1969.
11. Clark OH: Excess iodine intake and thyroid function and growth. Thyroid 1:69–72, 1990.
12. Block MA, Horn RC, Miller JM, et al.: Familial medullary carcinoma of the thyroid. Ann Surg 166:403–411, 1967.
13. Stoffer SS, Van Dyke DL, Bach JV, et al.: Familial papillary carcinoma of the thyroid. Am J Med Genet 25:775–783, 1986.
14. Favus MJ, Schneider AB, Stachura ME, et al.: Thyroid cancer occuring as a late consequence of head and neck irradiation. Evaluation of 1056 patients. N Engl J Med 294:1019–1025, 1976.
15. Oshima M, Ward MJ: Dietary iodine deficiency as a tumor promoter and carcinogen in male F334/NCr rats. Cancer Res 46:877–883, 1986.
16. McTiernan AM, Weiss NS, Daling JR: Incidence of thyroid cancer in women in realtion to reproductive and hormonal factors. Am J Epidemiol 120:423–435, 1984.
17. Ron E, Curtis R, Hoffman DA, et al.: Multiple primary breast and thyroid cancers. Br J Cancer 49:87–92, 1979.
18. Williams ED: The etiology of thyroid tumors. Clin Endocrinol Metab 8:193–207, 1979.
19. Kung TM, Ng WL, Gibson JB: Volcanoes and carcinoma of the thyroid: A possible association. Arch Environ Health 36:265–271, 1981.
20. Ron E, Kleinerman RA, Boice JD, LiVolsi VA, et al.: A population-based case-control study of thyroid cancer. J Natl Cancer Inst 79:1–12, 1987.
21. Clark OH, Gerend PL: Thyrotropin regulation of adenyl cyclase activity in human thyroid neoplasms. Surgery 97:539–546, 1985.
22. Schneider PB: TSH stimulation of ^{32}P incorporation into phospho-lipids of thyroids from patients with Graves' disease. J Clin Endocrinol Metab 38:148–150, 1974.
23. Duh QY, Clark OH: Factors influencing the growth of normal and neoplastic thyroid tissue. Surg Clin Noth Am 67:281–298, 1987.
24. Goretzki PE, Clark OH: Thyroid-stimulating hormone receptor studies. Prog Surg 19:181–204, 1988.
25. Querido A, Djokomoeljanto R, van Hardevelt C: The consequences of iodine deficiency for health. Presented at World Health Organization. PAHO., Washington DC, 1974.
26. Wynder EL: Some practical aspects of cancer prevention. N Engl J Med 246:573–582, 1952.

27. Bray GA: Increased sensitivity of the thyroid in iodine-depleted rats to the goitrogenic effects of thyrotropin. J Clin Invest 47:1640–1647, 1968.
28. Gaitan E, Nelson NC, Poole GV: Endemic goiter and endemic thyroid disorders. World J Surg 15(2):205–215, 1991.
29. Loebenstein BG, Buchan G, Sadeghi R, et al.: Transforming growth factor beta regulates thyroid growth. J Clin Invest 83:764–770, 1989.
30. Petenusci SO, Lopes RA, Silvanetto CR: Morphometric study of the reversibility of thyroid alterations in rats submitted to hypervitaminosis A. Int J Vitam Nutrit Res 50(3):238–241, 1980.
31. Taylor S: Physiologic consideration in the genesis and management of nodular goiter. Am J Med 20:698–709, 1956.
32. Gaitan, E: Goitrogens in food and water. Ann Rev Nutrit 10:21–39, 1990.
33. Dumont JE, Takeuchi A, Lamy F, et al.: Thyroid control: An example of a complex regulation network. Adv Cyclic Nucl Res 14:479–489, 1981.
34. Haslam SZ, Levely ML: Estrogen responsiveness of normal mouse mammary cells in primary culture: Association of mammary fibroblasts with estrogenic regulation of progesterone receptors. Endocrinology 116:1835–1840, 1985.
35. Duh QY, Clark OH: Growth factors for thyroid neoplasms. Prof Surg 19:205–222, 1988.
36. Siperstein AE, Miller RA, Clark OH: Stimulatory effect of vasoactive intestinal polypeptide on human normal and neoplastic thyroid tissue. Surgery 104:985–992, 1988.
37. Westermark K, Karlsson FA, Westermark B: Epidermal growth factor modulates thyroid growth and function in culture. Endocrinology 112:1680–1686, 1983.
38. Bishop JM: Viral oncogenes. Cell 42:23–36, 1985.
39. Varmus HE: The molecular genetics of cellular oncogenes. Ann Rev Genelt 18:553–612, 1984.
40. Weinberg RA: The action of oncogenes in the cytoplasm and nucleus. Science 230:770–776, 1985.
41. Arbeit JM: The molecular conspiracy in the mucosa: A review of the molecular biology of colorectal carcinogenesis. Prog Colon Rectal Surg 41:85–100, 1991.
42. Drucker BJ, Mamon HJ, Roberts TM: Oncogenes, growth factors and signal transduction. N Engl J Med 321:1381–1391, 1989.
43. Flier JS, Unverhill LH: Oncogenes and tumor-suppressor genes. N Engl J Med 318:618–622, 1988.
44. Wong RS, Passaro E, Jr.: Growth factors: Oncogenes and autocrine hypothesis. Surg Gynecol Obstet 168:468–473, 1989.
45. Koeffler HP, McCormick F: Molecular mechanisms of cancer. West J Med 155:505–514, 1991.
46. Greenspan FS: Radiation exposure. JAMA 237:2089–2092, 1979.
47. Alberts B, et al.: Basic genetic mechanisms. In: Alberts B, et al. (eds): Molecular Biology of the Cell. Garland, New York, 1989, pp 201–274.
48. Alberts B, Bray D, Lewis J: Control of gene expression. In: Alberts B, Bray D, Lewis J (eds): Molecular Biology of the Cell. Garland, New York, 1989, pp 201–274.
49. Baxter JD: Advances in molecular biology: Potential impact on diagnosis and treatment of disorders of the thyroid. Med Clin North Am 75:41–59, 1991.
50. Frauman AG, Moses AC: Oncogenes and growth factors in thyroid carcinogenesis. Endocrinol Metab Clin North Am 19:479–493, 1990.
51. Lemoine NR, Mayall ES, Wyllie FS, et al.: Activated ras oncogenes in human thyroid cancer. Cancer Res 48:4459–4462, 1988.
52. Lyons J, Lanois CA, Harsh G, et al.: Two g protein oncogenes in human endocrine tumors, Science 249:655–658, 1990.
53. Suarez HG, Villard JA, Caillou B, et al.: Gsp mutations in human thyroid tumors. Oncogene 6:677–679, 1991.
54. Coleta G, Cirafici AM, Vecchio G: Indications of the c-fos oncogene by throtropic

hormone in rat thyroid cells in culture. Science 253:458–460, 1986.
55. Dere WH, Hirayu H, Rapoport B: TSH and cAMP enhance expression of the myc proto-oncogene in cultured thyroid cells. Endocrinology 117:2452, 1985.
56. Aasland R, Lillenhaug JR, Male RJ, et al.: Expression of oncogenes in thyroid tumors: Coexpression of c-erb$_2$/neμ and c-erb. Br J Cancer 57(4):358–363, 1988.
57. Klimpfinger M, Ruhri C, Peutz B, et al.: Oncogene expression in a medullary thyroid carcinoma. Virchows Arch 54(4):256–259, 1988.
58. Reynolds RK, Hockzema GS, Vogel J, et al.: Multiple endocrine neoplasm induced by the promiscuous expression of a viral oncogene. Proc Natl Acad Sci USA 85:135–141, 1988.
59. Benedict WF, Xu H-J, Hu S, et al.: Role of the retinoblastoma gene in the initiation and progression of human cancer. J Clin Invest 85:988–993, 1990.
60. Weinberg RA: Tumor suppressor genes. Science 254:1138–1146, 1991.
61. Jackson CE, Norum RA, et al.: Linkage between MEN2b and chromosome 10 markers. Am J Human Gene 43:A17, 1988.
62. Larsson C, Skogseid B, Oberg K, et al.: Multiple endocrine neoplasms type 1 gene map to chromosome 11 and is lost in insulinoma. Nature 332:85–87, 1988.
63. Nelkin BD, Nakamura K, White RW, et al.: Low incidence of loss of chromosome 10 in sporadic and hereditary human medullary thyroid carcinoma. Cancer Res 9:4114–4117, 1989.
64. Sobel H, Narod SA, Nakamura Y, et al.: Screening for multiple endocrine neoplasia type 2a with DNA polymorphism analysis. N Engl J Med 321:996–999, 1989.
65. Brunt LM, Wells SA, Jr.: Advances in the diagnosis and treatment of medullary thyroid carcinoma. Surg Clinic North Am 67:263–279, 1987.
66. Duh Q-Y, Sancho JJ, Greenspan FS, et al.: Medullary thyroid carcinoma: The need for early diagnosis and total thyroidectomy. Arch Surg 124:1206–1210, 1989.
67. vanHeerden JA, Grant CS, Gharib H, et al.: Long term course of patients with persistent hypercalcitonemia after apparent curative primary surgery for medullary thyroid carcinoma. Ann Surg 212(4):395–401, 1990.
68. Bruns P.: Beobachtungen und Untersuchungen uber die Schilddrusen behandlung des Kropfes. Beitrz Klin Chir 16:521–534, 1896.
69. Greer MA, Astwood EB: Treatment of simple goiter with thyroid. J Clin Endocrinol Metab 13:1312–1331, 1953.
70. Shomaoka K, Sokal JE: Suppressive therapy of nontoxic goiter. Am J Med 57:576–583, 1974.
71. Crile G, Jr.: Endocrine dependency of papillary carcinomas of the thyroid. JAMA 195:101–104, 1966.
72. Thomas CG, Jr: Hormonal treatment of thyroid cancer. J Clin Endocrinol Metab 17: 232–240, 1957.
73. Cheung P, Lee JMH, Boey JH: Thyroxine suppressive therapy of benign solitary nodules: A prospective randomized study. World J Surg 13:818–825, 1989.
74. Morita T, Tamai H, Oshima A, et al.: Changes in serum thyroid hormone, thyrotropin and thyroglobulin concentrations during thyroxine therapy in patients with solitary thyroid nodules. J Clin Endocrinol Metab 69:227–230, 1989.
75. Gharib H, James EM, Charboneau JW, et al.: Suppressive therapy with leothyroxine for solitary thyroid nodules. N Engl J Med 317:70–75, 1987.
76. Berghout A, Wiersinga WM, Drexhage HA, et al.: Comparison of placebo with L-thyroxine alone or with carbinazole for treatment of sporadic non-toxic goitre. Lancet 336:193–197, 1990.
77. Dunhill TP: The surgery of the thyroid gland (The Lettsomian Lectures). Trans Med Soc London 60:234–237, 1937.
78. Balme HW: Metastatic carcinoma of the thyroid successfully treated with thyroxine. Lancet 1:812–819, 1954.

79. Purves HD, Griesbach WE, Kennedy TH: Studies in experimental goiter: Malignant change in transplanted rat thyroid tumor. Br J Cancer 5:301–306, 1951.
80. Nichols CW, Jr., Lindsay S, Sheline GE, Chaikoff IL: Induction of neoplasms in rat thyroid glands by x-irradiation of a single lobe. Arch Pathol Lab Med 80:177–183, 1965.
81. Doniach I, Wiliams ED: The development of thyroid and pituitary tumors in the rat two years after partial thyroidectomy. Br. J Cancer 16:222–230, 1962.
82. Goldberg RC, Lindsay S, Nichols CW, Jr., Chaikoff IL: Induction of neoplasms in the thyroid gland of the rat by subtotal thyroidectomy and by injection of one microcurie of I^{131}. Cancer Res 24:35–44, 1964.
83. Fogelfeld L, Wiviott MBT, Shore-Freedman E, et al.: Recurrence of thyroid nodules after surgical removal in patients irradiated in childhood for benign conditions. N Engl J Med 320:835–840, 1989.
84. Razack MS, Shimaoka K, Rao U: Suppressive therapy of thyroid nodules in patients with previous radiotherapy to the head and neck. Am J Surg 156:290–293, 1988.
85. DeGroot LJ, Reilly M, Pinnemenini K, Refetoff S: Retrospective and prospective study of radiation-induced thyroid disease. Am J Med 74:852–862, 1983.
86. Dralle H, Bocker KW, Dohler KD, et al.: Growth and function of thirty-four human benign and malignant thyroid xenografts in untreated nude mice. Cancer Res 45:1239–1245, 1985.
87. Grant S, Luttrell B, Reeve T, et al.: Thyroglobulin may be undetectable in the serum of patients with metastatic disease secondary to differentiated thyroid carcinoma. Cancer 54:1625–1628, 1984.
88. De Rubertis, Yamashita FK, Dekker A, et al.: Effects of thyroid stimulating hormone on adenylate cyclase activity and intermediary metabolism of 'cold' thyroid nodules and normal human thyroid tissue. J Clin Invest 31:1109–1117, 1972.
89. Clark OH: TSH suppression in the management of thyroid nodules and thyroid cancer. World J Surg 5:39–47, 1981.
90. Smeds S, Peter HJ, Gerber H, et al.: Effects of thyroxine on cell proliferation in human multidodular goiter. A study on growth of thyroid tissue transplanted to nude mice. World J Surg 12:241–245, 1988.
91. Dumont JE, Roger PP, Ludgate M: Assays for thyroid growth immunoglobulins and their clinical implications: Methods, concepts, and misconceptions. Endocrinol Rev 8:448, 1987.
92. McKenzie JM, Zakarija M: Clinical review 3, The clinical use of thyrotropin receptor antibody measurements. J Clin Metab. 69:1093–1099, 1989.
93. Filetti S, Belfiore A, Amir SM, et al.: The role of thyroid-stimulating antibodies of Graves' disease in differentiated thyroid cancer. N Engl J Med 318:753–755, 1988.
94. Laurent E, Van Sande J, Ludgate M, et al.: Unlike thyrotropin, thyroid stimulating antibodies do not activate phospholipase C in human thyroid cells. J Clin Invest 87:1634–1642, 1991.
95. Kawabe Y, Eguchi J, Shimomura C, et al.: Interleukin-1 production and action in thyroid tissue. J Clin Endocrinol Metab 68:1174–1179, 1989.
96. Mime M, Tramontano D, Chin WW, et al.: Interleukin-1 stimulates thyroid cell growth and increases the concentration of the c-myc proto-oncogene RNA in thyroid follicular cells in culture. Endocrinology 120:1212–1217, 1987.
97. Zakarija M, McKenzie JM: Influence of cytokines on growth and differentiated function of $FRTL_5$ cells. Endocrinology 125:1260–1264, 1989.
98. Heldin NE, Cvejl CD, Smeds S, Westermark B: Coexpression of functionally active receptors for thyrotiopin and platelet derived growth factor in human thyroid carcinoma cells. Endocrinology 129(4):2187–2193, 1991.
99. Heldin NE, Gustavsson G, Claesson-Welsh L, et al.: Aberrant expression of receptors for platelet-derived growth factor in an anaplastic thyroid carcinoma cell line. Proc Math Sci USA 85:9302–9304, 1988.
100. Roger PP, Dumont JE: Factors controlling proliferation and differentiation of canine

thyroid cells cultured in reduced serum conditions: Effects of thyrotropin, cyclic AMP and growth factors. Mol Cell Endocrinol 36:79–93, 1984.
101. Terrier P, Sheng Z-M, Schlumberger M, et al.: Structure and expression of c myc and c fos proto-oncogenes in thyroid carcinomas. Br J Cancer 57:43–50, 1988.
102. Lemoine NR, Wyllie FS, Thurston V, et al.: Ras oncogene activitation: An early event in human thyroid tumor genesis. Ann Endocrinol 49:191–197, 1988.
103. Wyllie FS, Lemoine NR, Williams ED, Thomas-Wynnford D: Structure and expression of nuclear oncogenes in multi-stage thyroid tumor genesis. Br J Cancer 60(4):561–565, 1989.
104. Lemoine NR, Mayall ES, Wyllie FS, et al.: High frequency of ras oncogene activation in all stages of human thyroid tumorigenesis. Oncogene 4(2):159–164, 1989.
105. Namba H, Rubin SA, Fagin JA: Point mutations of ras oncogenes are an event in thyroid tumorigenesis. Mol Endocrinol 4:1474–1479, 1990.
106. Namba H, Gutman RA, et al.: H ras proto-oncogene mutations in human thyroid neoplasms. J Clin Endocrinol Metab 71:223–229, 1990.
107. Duh QY, Zhang Y, Lyons JF, et al.: Ras oncogene activation in human thyroid tumors is associated with high adenylate cyclase response to thyrotropin (TSH) Pressented at The Endocrine Society Annual Meeting, June 20–23, 1990.
108. Schark C, Fulton N, Jacoby RF, et al.: N-ras 61 oncogene mutations in Hurthle cell tumors. Surgery 108(6):994–999, 1990.
109. Stringer BM, Rocoson JM, Pprkar MD, et al.: Detection of the H-ras oncogene in human thyroid neoplastic carcinomas. Experientia 45(4):372–376, 1989.
110. Karga H, Lee J-K, Vickery AL, et al.: Ras oncogene mutations in benign and malignant thyroid neoplasms. J Clin Endocrinol Metab 73:832–836, 1991.
111. Wright PA, Williams ED, Lemoine NR, Thomas-Wynford D, et al.: Radiation-associated and 'spontaneous' human thyroid carcinomas show a different pattern of ras oncogene mutation. Oncogene 6:471–473, 1991.
112. Johnson TL, Lloyd RV, Thor A: Expression of ras oncogene p 21 antigen in normal and proliferative thyroid tissues. Am J Pathol 127:60–65, 1987.
113. Pacini F, Basolo F, Fugazzola U, et al.: Expression the ras gene product (p21) in primary and metastatic differentiated thyroid cancer: Possible predictor of poor outcome. Presented at 19th ETA Meeting, Hannover, 1991.
114. Nakagawa T, Mabry M, de Bustros A, et al.: Introduction of v-Ha-ras oncogene induces differentiation of cultured human medullary thyroid carcinoma cells. Proc Natl Acad Sci USA 84:5923–5927, 1987.
115. Bar-Sagi D, Feramisco JR: Microingestion of the ras oncogene product into PC12 cells induces morphologic differentiation. Cell 42:841–848, 1985.
116. Shi Y, Zou M, Schmidt H, et al.: High rates of ras codon 61 mutation in thyroid tumors in an iodide-deficient area. Cancer Res 51:2690–2693, 1991.
117. Goretzki PE, Stacy-Phipps S. Roseneau W, et al.: Heterogeneous distribution of Gsp oncogenes in human thyroid tumors. Nature, submitted.
118. Lidereau R: Molecular characterization of the genetic risk in breast cancer. Eur J Gynecol Oncol 10(3):182–184, 1989.
119. Muschel RJ, Williams JE, Lowy DR, Liotta LA: Having ras induction of metastatic potential depends upon oncogene activation and the type of recipient cell. Am J Pathol 121:1–8, 1985.
120. O'Sullivan C, Barton CM, Staddon SL: Activating point mutations of the gsp oncogene in human thyroid adenomas. Mol Carcinog 4(5):345–349, 1991.
121. Goretzki PE, Lyons J, Demeure M, et al.: Biological important differences in the oncogene pattern of differentiated thyroid cancer from German and American patients. World J Surg, 116:576–580, 1992.
122. Weinstein LS, Shenker A, Gejman PV, et al.: Activating mutations of the stimulatory protein in the McCune-Albright syndrome. N Engl J Med 325(24):1688–1695, 1991.
123. Fusco A, Grieco M, Santoro M, et al.: A new oncogene in human thyroid papillary

carcinomas and their lymph-node metastases. Nature 328:170–177, 1987.
124. Santoro MR, Rosati R, Grieco M, et al.: The ret proto-oncogene is consistently expressed in human pheochromocytomas and thyroid medullary carcinomas. Oncogene 5(10):1595–1598, 1990.
125. Dongi R, Soezi G, et al.: The oncogene associated with human papillary thyroid carcinoma (PTC) is assigned to chromosome 10q11-q12 in the same region as multiple endocrine neoplasia type 2A. Oncogene 4(41):521–523, 1990.
126. Bargarzone I, Pierotti MA, Monzini N, et al.: High frequency of activation of tyrosine kinase oncogenes in human papillary thyroid carcinoma. Oncogene 4(12):1457–1462, 1989.
127. Wright PA, Lemoine NR, Goretzki PE, et al.: Mutation of the p53 gene in a differentiated human thyroid carcinoma cell line, but not in primary thyroid tumors. Oncogene 6:1693–1697, 1991.
128. Ito T, Seyama T, Mizuno T, et al.: Unique association of p53 mutations with undifferentiated but not with differentiated carcinomas of the thyroid gland. Cancer Res 52: 1369–1371, 1992.
129. Vogelstein B, Fearon ER, Hamiton SR, et al.: Genetic alterations during colorectal tumor development. N Engl J Med 319:525–532, 1988.
130. Mukhopadhyay T, Tainsky M, Cavender AC, Roth JA: Specific inhibition of K ras expression and tumorigenicity of lung cancer cells by antisense RNA. Cancer Res 51:1744–1748, 1991.

5. Growth regulation of human neuroblastoma

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Introduction

Neuroblastoma is a highly malignant tumor of infants and children. It typically occurs before the age of 5 years and accounts for up to 50% of all malignancies among infants [for review see 1,2]. A significant fraction of cases are identified neonatally, indicating that the tumor can arise during fetal life and may represent a disorder of normal development [3]. These tumors arise in sympathetic neuroblasts that originate in the neural crest and are destined to become chromaffin or neuronal tissues of the peripheral nervous system [4]. Sixty-five percent of neuroblastomas occur in the abdomen, where adrenal medullary tumors account for 40% of these tumors [1,2]. Approximately 50% of infants and 70% of older children with neuroblastomas have evidence of tumor spread beyond the primary location to metastatic sites, including the lymph nodes, bone, bone marrow, liver, and skin, at the time they first come to medical attention [1]. In children under 1 year of age, a special presentation of disseminated neuroblastoma, which is most clearly distinguished from the more common presentation of advanced-stage neuroblastoma in that it does not involve lytic lesions of the bone, has been recognized [5–8]. This group, designated stage IVs, includes approximately 17% of neuroblastoma tumors arising in children under the age of 1 year. Remarkably these tumors regress without therapy, while those of older patients or of young children with metastatic disease to bone have a very poor prognosis [1].

Neuroblastomas may be distinguished from other histologically similar small, round blue cell tumors, such as neuroepithelioma and Ewing's sarcoma, by patient age, urinary excretion of the catecholamine metabolites homovanillic acid (HVA) and vanillylmandelic acid (VMA), absence of a t(11,22) chromosomal alteration [9], and secretion of neurotransmitter biosynthetic enzymes [for review see 10]. Neuroblastomas are also characterized by greatly diminished or absent HLA Class I antigen expression, abnormalities of chromosome 1, and *src* and *ras* oncogene expression, and may contain multiple copies of N-*myc* oncogene DNA (see below). Moreover, recent investigations indicate that expression of the *ret* oncogene

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may be specific to neuroblastomas [11,12], while expression of *dbl* may be specific to Ewing's sarcomas [13].

Chromosomal abnormalities

Karyotypic abnormalities in tumor cells of patients with neuroblastoma have been documented in many studies [14–20]. These include homogeneously staining regions (HSRs), double-minute chromosomes (DMs), and chromosomal alterations, such as deletions and aneuploidy.

HSRs and DMs

HSRs and DMs are anomalous nonbanding chromosomal material present in metaphase preparations of many human neuroblastoma tumors. These structures were first identified in neuroblastoma. Most commonly they were found in tumors from patients with advanced-stage disease (stage III and IV) who responded poorly to treatment [17,20–24]. HSRs and DMs are often present in cells resistant to the cytotoxic effect of high-dose chemotherapeutic agents [24]. It is known that these structures contain genes that encode drug resistance, and it is possible that the genes present in DMs and HSRs of tumors confer a selective growth advantage on those tumor cells as well. HSRs and DMs have since been detected in many other human tumor types, including colon carcinoma, small cell lung carcinoma, and retinoblastoma [25–27].

The N-*myc* oncogene (see below), located on chromosome 2 [28], is often amplified in HSRs and DMs, where it is overexpressed [28–33]. This finding suggests that these chromosomal regions contain amplified genes that contribute to the pathogenesis of neuroblastomas. The genes amplified in HSRs and DMs are not thought, however, to initiate neuroblastomas, as they are detected only in advanced-stage tumors. The role of such genes in the progression or metastasis of neuroblastomas has not been resolved, although amplification of N-*myc* is associated with a poor prognosis (see below).

Chromosomal deletions: 1p, 14q, 11q

Chromosomal deletions have been detected in many neuroblastomas. Most notable among these are deletions in chromosomes 1p, 14q, and 11q. Allelic loss in chromosome 14q has been reported in 10 of 21 (48%) [34] and 4 of 12 (33%) [35] primary tumors from patients with neuroblastoma. The significance of 14q deletions, however, is not clear, as such losses are infrequent among neuroblastomas. Also, they may be detected in tumors from patients with all stages of disease, as well as in patients with ganglioneuroblastoma, a form of neuroblastoma that has a favorable prognosis [34]. Deletions in chromosome 11q have been reported in tumor DNA from 5 of 12 (42%)

patients evaluated [35]. Allelic loss in 11q has been detected in tumors other than neuroblastomas, suggesting that a tumor suppressor gene important for several human tumors may be located in this region [35].

One of the most frequent chromosomal alterations observed in neuroblastoma is the apparent loss of a portion of the short arm of chromosome 1. Such alterations were originally observed in neuroblastoma cell lines isolated from patients with advanced-stage disease [19,20,36] and may differ from 1p deletions observed in tumor cells of other cancers [37]. The reported frequency of $1p^-$ among neuroblastomas is high: 65% of neuroblastoma cell lines and 70–100% of advanced-stage tumors have this change [22,23,38, 39]. Christianson [23] detected chromosome 1 alterations in 15 of 18 patients with stage IV neuroblastoma, 2 of 3 patients with stage III, and 0 of 7 patients with stages I, II, and IVs. All of the seven patients with stages I, II, or IVs without chromosome 1 abnormalities were alive with no evidence of disease. Life table analyses revealed that there was a 90% probability of survival for patients lacking 1p abnormalities compared to <10% in patients with detectable 1p alterations. These findings suggest that structural changes in chromosome 1 may be used to discriminate between patients with good and bad prognoses [23].

The frequency of chromosome 1 alterations and the association of such alterations with poor clinical outcome has raised the possibility that such changes may be associated with the inactivation of a tumor suppressor gene [3,37,40] or the activation of a neuroblastoma susceptibility gene [39]. Support for such a hypothesis comes from studies demonstrating the suppression of the ability of neuroblastoma tumor cells with chromosome 1 alterations to form tumors after fusion with Hela carcinoma cells [41]. Cytogenetic and molecular studies of tumor samples using chromosome-specific polymorphic DNA markers indicate that the extent of deleted material on chromosome 1 varies greatly between tumors [37,39]. However, the region most commonly involved includes 1p31 to ter [19,42–44]. Using molecular genetic analysis Fong reported that the most distal breakpoint noted is at or near the FGR locus, 1p34–36 [43]. Using a region-specific panel of probes generated by microdissection and microcloning, Schwab [37] and Weith [45] have identified a region, 1p36.1–2, which is deleted in more than 90% of stage III and IV neuroblastoma tumors.

Ploidy: Near triploid versus near diploid DNA index

In addition to the predictive value of $1p^-$, a strong correlation has been observed between DNA ploidy and prognosis among patients with neuroblastoma [23,46–53]. In one study, Kaneko [50] observed that near diploid or pseudodiploid and hypotetraploid karyotypes with numerical and structural abnormalities were associated with chromosome 1 alterations, HSRs, DMs, and N-*myc* amplification. Such features were observed mostly in patients greater than 1 year of age with stage III or IV neuroblastoma. The

mean survival time for these patients was 376 days, with 7 of 11 of these patients succumbing to the disease. A near-triploid karyotype, on the other hand, was associated with almost complete haploid sets, few structural abnormalities, and no N-*myc* amplification. Such characteristics were found only in infants with stage I or II disease, who were reported to be alive with no evidence of disease. Bourhis [52] also reported that the combination of ploidy and N-*myc* status can be highly predictive of outcome. In these studies the 2-year actuarial disease-free survival rate was 94% for patients with near-triploid neuroblastoma (DNA index between 1.25 and 1.7), compared to 11% and 45% for patients with near-diploid tumors, with and without N-*myc* amplification, respectively ($p < .01$).

Oncogenes

Proto-oncogenes are normal cellular genes whose aberrant expression contributes to the development of tumors [54]. Although expression of such genes may have prognostic value, there is presently limited evidence implicating the expression of specific oncogenes with tumor initiation or progression of neuroblastomas. The *ret* oncogene, which encodes two isoforms of a receptor type tyrosine kinase [55,56], has been shown to be expressed in 11 of 11 neuroblastoma cell lines and all 29 neuroblastoma tumors examined. Expression has not been detected in a large number of other tumors or in 19 nonneuroblastoma solid tumors and in one human diploid fibroblast cell line [11,12]. The levels of mRNA encoding *ret* varied up to 100-fold in these tumors. Such expression also appeared to be independent of patient age, stage, histological grade, N-*myc* amplification or expression, or the serum level of neuron-specific enolase [11,12]. Since other neural-crest-derived tumors do not express *ret* [57], expression of this gene may be related to its expression in neuroblasts in which neuroblastoma arises [58]. No structural rearrangement or amplification of *ret* has been detected in any neuroblastomas [11].

c-*src*, the cellular homologue of the viral transforming gene, v-*src* is a tyrosine-specific kinase [59,60] that is expressed at low levels in most cell types. Elevated levels of c-*src* kinase activity are detected in neurons and chromaffin cells, as well as in some other cell types [61–63]. High levels of c-*src* mRNA or protein have also been detected in many human tumors, including small cell lung carcinoma cell lines, colon carcinoma, rhabdomyosarcoma, breast cancer, and neuroblastoma but not in any other childhood tumors [64–70]. The expression of additional forms of c-*src*, c-*src*-NI, and c-*src*-NII appears to be limited to neuroblasts, neurons, and cells differentiated along a neuronal lineage [61,70–72]. Thus, c-*src*-N may be a marker for neuroblastoma among small, round blue-cell tumors. Among neuroblastoma, c-*src*-N is highly expressed in less-aggressive, low-stage, more-differentiated, good-prognosis tumors of infants, but not in tumors

from older, poor-prognosis neuroblastoma patients [73,74]. Expression of c-*src* in neuroblastomas also may be inversely correlated with N-*myc* expression [74].

The *ras* oncogene, which functions in the transduction of signals across the cell membrane [75–78], has also been shown to be expressed at elevated levels in neuroblastoma of young children, which has a favorable prognosis, in ganglioneuroblastoma, and in neuroblastomas differentiated by chemotherapy [79,80]. The *ras* gene is also expressed at high levels in normal rat brain and has been shown to promote morphological differentiation and growth inhibition of rat pheochromocytoma PC-12 cells into neuronlike cells [81–84]. These findings suggest that *ras* may have an important function in neural tissues [79]. A single point mutation in the gene coding for *ras* is sufficient to transform cells. An analysis of DNAs from 10 neuroblastoma cell lines for mutations in N-*ras*, H-*ras*, or K-*ras* revealed no point mutations, except for a single point mutation in codon 59 of N-*ras* in DNA from SK-N-SH. This mutation was postulated to result from in vitro passage as early passages or sublines of SK-N-SH did not contain such a rearrangement [85].

Much has been written regarding the association of N-*myc* oncogene amplification and the pathogenesis of neuroblastomas. N-*myc* was originally identified in an amplified form in 8 of 9 neuroblastoma cell lines and in one tumor by sequence similarity to c-*myc* [21,28,29], which was previously shown to be amplified in cell lines from acute promyelocytic leukemia [86], colon carcinoma [25], and lung tumor [26]. By virtue of its homology with c-*myc* and by its amplification in a majority of neuroblastoma cell lines, N-*myc* was thought to contribute directly to tumorigenesis. Support for such a hypothesis was provided by transfection studies, which indicated that N-*myc* can support mutationally activated *ras*-H in the tumorigenic conversion of primary rat embryo cells [87,88]. Subsequent reports revealed that N-*myc* was not amplified in all neuroblastomas. Rather, multiple copies of N-*myc* were detected in only 30–40% of all neuroblastomas, primarily in tumor tissues from patients with either advanced-stage disease [30,89,90], disease originating in the suprarenal region [91], histologically undifferentiated tumors [91–93], surgically unresesictable primary tumors [94,95], and tumors associated with low levels of urinary VMA and HVA [96,97]. N-*myc* amplification was rarely observed in tumors presenting as stage I, II, or IVs [31,89,90,98]. Such findings lead to the suggestion that rather than representing the primary transformation event in neuroblastomas, N-*myc* may be associated with progression of the disease [90].

More recent studies reveal that, in addition to stage III and IV neuroblastoma, N-*myc* may be detected in an amplified form in some early stage tumors, including IVs neuroblastomas [92,99–103]. N-*myc* amplification is associated with a poor prognosis, independent of patient age or disease stage at presentation [92,100]. Among neuroblastoma cell lines that express N-*myc*, the gene is expressed only in the round neuritic 'N' cells, which grow

in soft agar and are tumorigenic in athymic mice. N-*myc* is not expressed in the flat epithelial-like component 'S' cells of neuroblastoma cell cultures. These cells do not grow in soft agar and are not tumorigenic in nude mice [104].

The N-*myc* oncogene encodes two nuclear DNA binding phosphoproteins [105] with molecular weights of 65 and 67 kDa [106], and 62/64 [107]. These proteins have a short half-life and resemble proteins encoded by the c-*myc* oncogene [108]. N-*myc* expression is developmentally regulated and is found in immature B and T lymphocytes, as well as fetal tissues such as brain, retina, lung, kidney, and cranial muscle. Several different tumors of neuroendocrine origin, such as neuroblastoma, retinoblastoma, and small cell lung carcinoma [29,30,109–118], also express N-*myc*. In human fetal brain, N-*myc* mRNA is detectable in mitotic undifferentiated neural cells but not in differentiated neurons [113]. Neuroblast precursor cells express N-*myc* while migrating from the neural crest, while proliferating, and while invading the fetal adrenal or sympathetic ganglia. N-*myc* expression ceases, however, once the neuroblasts are established in the adrenal gland [112]. By virtue of its DNA binding capacity, as well as by the presence of conserved motifs previously identified in transcription factors, the N-*myc* gene is thought to encode a regulatory protein. In addition, its transient and localized expression during early stages of organogenesis suggests that N-*myc* may regulate gene expression in the developing organism [119].

N-*myc* expression has been postulated to correlate with increased growth potential [120,121]. However, many investigators believe that such expression is associated with the differentiated state of the cells [110,112,119,122–124]. Agents that induce differentiation of neuroblastoma cells have also been shown to reduce N-*myc* expression [125–127]. Studies comparing the growth characteristics of neuroblastoma cells in nude mice, however, indicate that there is no difference in tumorigenecity between neuroblastoma IMR-32 cells, which contain amplified copies of N-*myc*, and SK-N-SH cells, which contain a single copy N-*myc* [128].

Class I HLA expression

One of the mechanisms important for tumor growth and evasion of the host immune system may involve altered expression of class I major histocompatibility complex (MHC) antigens. These membrane molecules are important for the presentation of tumor antigens to cytotoxic T lymphocytes, which is key for the in vivo destruction of tumor cells. In mice, loss of class I antigen expression has been observed in both spontaneous tumors, such as leukemias and sarcomas [129–131], and in virus-induced tumors [132]. Reversion to the benign phenotype has been observed following transfection of neuroblastoma cells with class I genes, which restore normal MHC antigen levels and sensitivity to cytotoxic T-lymphocytes [133–135]. In human tumors a reduction of HLA-A, -B, and -C antigens has been demon-

strated in many neoplasms, including choriocarcinoma; teratocarcinoma; skin carcinoma; small cell lung carcinoma; colorectal, laryngeal, and breast carcinomas; melanoma; lymphoma; and neuroblastoma [for review, see 136].

Among cell lines derived from human neuroblastomas or from tumors of neuronal origin, class I HLA expression has been shown to be very low or absent [137,138]. These findings suggest that such expression may lead to the escape of neuroblastoma tumor cells from immunosurveillance [139, 140]. One study examining such a possibility did not demonstrate a correlation between the loss of HLA expression and the prognosis of patients with neuroblastoma [141]. Using the monoclonal antibody W6/32 and anti-β-2-microglobulin, Cooper demonstrated that MHC antigen expression is developmentally regulated in the human adrenal gland [142]. It is undetectable in adrenal neuroblasts from human fetuses estimated to be at 24 weeks of gestation, but is detectable in postnatal adrenal neuroblast cells. Thus MHC antigen expression among neuroblastomas may reflect the stage during development at which individual tumors arise.

Surface expression of MHC antigens in neuroblastoma has been induced by treatment with gamma-IFN [143–147] as well as by other agents [145]. A phase I/II trial using gamma-IFN in patients with neuroblastoma has indicated the relative safety of the treatment, but has not resulted in significant clinical responses [148]. Differentiation concomitant with chemotherapy in primary as well as metastatic sites has also resulted in enhanced expression of class I antigens [138].

Examination of the expression of class I HLA and N-*myc* genes in neuroblastoma cell lines suggests that an inverse relationship exists between the expression of N-*myc* and HLA class I molecules [149]. Transfection of N-*myc* into neuroblastomas [150] or c-*myc* into melanomas [151] has also been shown to modulate the expression of HLA class I molecules. Furthermore, fusion of N-*myc* amplified IMR-32 cells with L cells resulted in the downregulation of N-*myc* gene expression and the upregulation of HLA expression [152]. These findings suggest that N-*myc* overexpression may result from inactivation of a suppressor gene, while downregulation of N-*myc* reactivates HLA expression [152]. Two reports, however, present data that do not support the notion that expression of N-*myc* and HLA genes are linked, one using N-*myc* transfection [153] and the other employing cytokine treatment [147]. These studies did not report a close association between the expression of N-*myc* and HLA, and indicate that an understanding of the precise relationship between these genes will require further study.

Autocrine and paracrine growth

It has been proposed that the growth of many human tumors may be mediated by soluble factors produced by the tumor cells themselves [154]. Critical data to support this hypothesis, however, are available for only a

few types of tumors [155–157]. On the other hand, a significant literature exists to support the possibility that the growth of human tumor cells may be stimulated by factors produced by nonmalignant cells within the tumor [e.g., 158,159]. Such a mechanism is consistent with the paracrine model of tumor growth.

Exogenous factors that stimulate the growth of neuroblastomas in vitro

Studies using serum-containing medium have suggested that epidermal growth factor (EGF) or the combination of EGF and hydrocortisone stimulate the proliferation of substrate-adherent, S-type, neuroblastoma cells [160–162] in semisolid medium [163]. EGF has also been reported to stimulate the proliferation of 7 of 8 neuroblastoma cell lines in liquid medium containing low concentrations of serum [164], and of SK-N-BE and CA-2 neuroblastoma cells in semisolid medium. The range of this stimulation was 1.3- to 3.5-fold 14 days after growth factor addition. Basic fibroblast growth factor (bFGF), nerve growth factor (NGF), and cell-free extract from selected embryonic chick eye tissue (CIPE) have also been reported to stimulate the proliferation of IMR-32 neuroblastoma cells in serum-containing medium [165].

In a chemically defined serum-free medium, insulin and insulinlike growth factor (IGF) I or II stimulated the proliferation of almost all human neuroblastoma cell lines, whereas the addition of other such factors, including EGF, acidic FGF, bFGF, platelet-derived growth factor, endothelial cell growth factor, granulocyte-macrophage colony stimulating factor, and transforming growth factor beta, had no effect [166–169]. Insulin stimulated the growth of human neuroblastoma cells only when supraphysiological concentrations (1–10 μg/ml) of the peptide were used [167,168]. In contrast, physiological concentrations of IGF I or II, 2–10 ng/ml, stimulated the growth of all neuroblastoma cell lines studied. The proliferation of three such cell lines was stimulated six- to sevenfold using IGF-II for 9 days [167] (Fig. 1).

Insulin, IGF-I, and IGF-II stimulate growth by binding to the same receptor, the type I IGF receptor [170–172], which is present on the cell surface of all neuroblastoma cell lines studied to date [166,173–176]. Since supraphysiological concentrations of insulin are required to stimulate the growth of neuroblastoma cells, and physiological concentrations of IGF I or II have the same effect, it seems likely that a physiological ligand mediating the growth of neuroblastomas in vivo will be an IGF rather than insulin itself (see below).

Factors produced by tumor cells that mediate the growth of neuroblastomas

Several studies have examined the possibility that cell extracts or cell-conditioned medium contains factors mitogenic for neuroblastoma cells. In

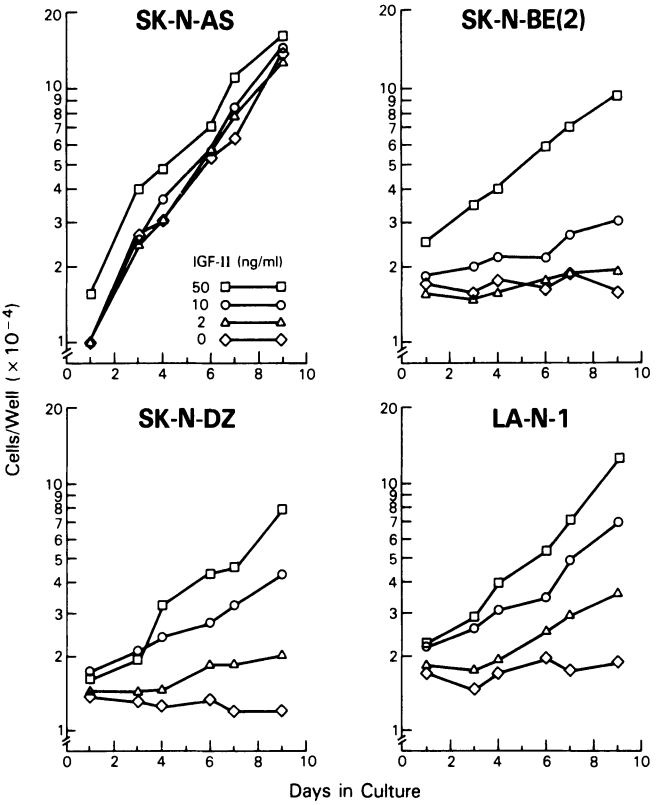

Figure 1. IGF-II stimulates the proliferation of human neuroblastoma cells in vitro. 10^4 human neuroblastoma cells were plated ($3 \times 10^4/cm^2$) into 96-well tissue culture dishes containing serum-free N2E medium [166] with or without IGF-II. The cells were refed on day 6. Cell number was determined on the indicated days using the MTT assay. (Reproduced with permission [167].)

one such report medium conditioned by the human neuroblastoma cell line BE(2)-C was shown to contain a factor that stimulated its own growth, as well as that of other neuroblastoma cells. Biochemical analysis of this factor suggested that it may be IGF-II [168]. Chelmicka-Schorr et al. [177] have reported that a factor produced by PC-12 pheochromocytoma cells augments the growth of mouse C-1300 neuroblastoma cells. A factor elaborated in newborn sympathetic ganglion cells has a similar effect. Ablation of the sympathetic nervous system in mice by chemical treatment prior to C-1300 injection also suppressed growth of the tumor significantly [178]. Based upon studies indicating that virtually all pheochromocytomas express high levels of mRNA encoding IGF-II [166], as well as other studies indicating that neuroblastomas are growth stimulated by this peptide, it is likely that the factor made by PC-12 cells, which is mitogenic to mouse C-1300 neuroblastoma cells, will be IGF-II. Similarly, the factor produced by

sympathetic ganglion cells, which enhances C-1300 tumorigenicity, may also be IGF-II.

IGF-II mediated autocrine and paracrine growth of neuroblastomas

An analysis of growth factor gene expression in neuroblastomas indicated that some neuroblastoma cell lines (6 of 22) and tumors (2 of 8) express mRNA encoding IGF-II [166,167]. One such cell line, SK-N-AS, which expresses high levels of IGF-II mRNA, is capable of continuous growth for many months in cell culture medium without serum or exogenous growth factors (Fig. 1). These cells synthesize biologically active IGF-II, display type I IGF receptors on their cell surface, and their growth in mitogen-free medium may be blocked using antibodies that bind the type I IGF receptor [166]. These studies indicate that SK-N-AS neuroblastoma cells are capable of autocrine growth mediated by endogenously produced IGF-II, and suggest that the growth of other neuroblastoma cell lines and tumors that synthesize IGF-II and express the IGF receptor may be autocrine as well. A survey of the growth characteristics of 25 other human neuroblastoma cell lines revealed that most of these cell lines grow well in serum-free medium containing insulin or IGF-II (Fig. 1). Elimination of insulin from the culture medium resulted in the sluggish growth of most such cell lines, even among some cell lines that expressed high (e.g., SMS-KAN or SK-N-FI) or moderate levels (CHP-238 or SK-N-BE-[2]) of IGF-II. The growth of many such cell lines in mitogen-free medium was improved, however, if the cell density was increased, suggesting that these cells may synthesize low levels of paracrine-acting growth factors.

Temporal expression of IGF-II in the human adrenal gland

IGF-II is thought to function primarily in the fetus [179–186], whereas IGF-I is thought to function during adolescence [187,188]. Since neuroblastomas, which are thought to originate during fetal development, are growth stimulated in vitro by IGF-II, we examined the expression of IGF-II in the human adrenal gland, the tissue in which many neuroblastomas arise [4]. Using in situ hybridization and an antisense oligonucleotide probe specific for IGF-II [189], we detected IGF-II mRNA expression in adrenal cortical cells at all times during fetal development, but not in adrenal cortical cells of newborns or adults (Fig. 2) [167]. Several reports have indicated that IGF-II mRNA expression declines dramatically in adrenal cortical cells from the high levels expressed at approximately 8–9 weeks of gestation to low levels at 24 weeks of gestation and to undetectable levels after birth and in adulthood [167,181,189–192].

The temporal expression of IGF-II in adrenal medullary tissue is more difficult to determine. During development adrenal neuroblast cells invade the primordial adrenal gland and populate it to form the adult adrenal

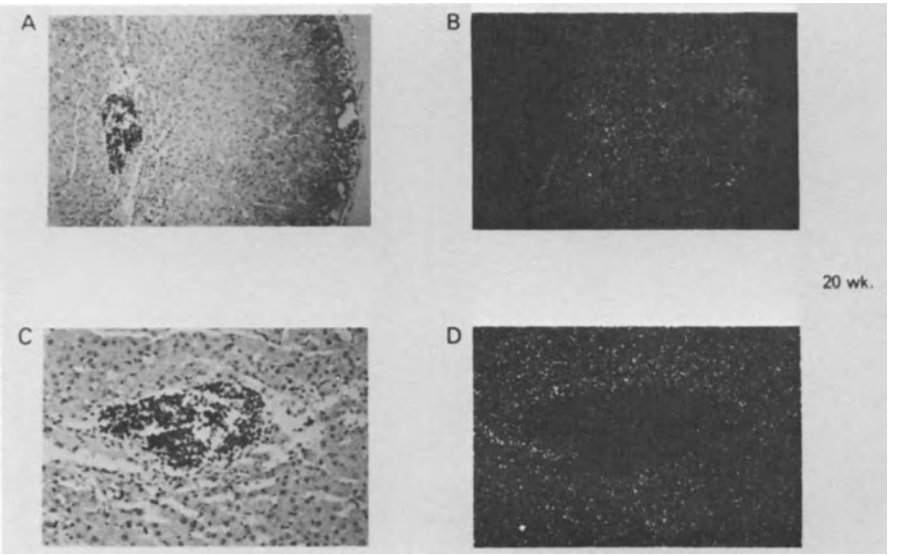

Figure 2. Expression of IGF-II in the human fetal adrenal gland at 20 weeks gestation. Human adrenal gland tissues were evaluated for IGF-II gene expression by in situ hybridization histochemistry using an oligonucleotide probe specific for IGF-II [166,167,189]. Panels B and D are dark field images of the same views shown in panels A and C. IGF-II positive cells (white dots in panels B and D) are *within adrenal cortical tissue*. IGF-II negative cells in the center of the field are adrenal medullary precursor cells [142].

medulla [193,194]. At early times such cells are few in number. However, when such cells were identified they did not express detectable IGF-II [167] (Fig. 2). IGF-II mRNA expression was also not detected in adrenal medullary cells of adults using in situ hybridization, although low levels were found using Northern blot analysis [166,167]. These data indicate that the adult adrenal medulla expresses low levels of IGF-II. It remains to be determined whether fetal adrenal neuroblasts also express low levels of IGF-II mRNA that are currently undetectable using in situ hybridization.

IGF-II gene expression and the proliferation of neuroblastomas in vivo

IGF-II gene expression has been detected in both parenchymal and stromal tissue of many different human embryonic organs and tumors [112,158, 159,181,189,195]. Using an antibody directed against rat IGF-II, Suzuki [196] detected IGF-II protein in 3 of 4 ganglioneuroblastomas and in adult adrenal medulla. An examination of IGF-II mRNA expression in neuroblastoma tumor tissues using in situ hybridization has indicated that IGF-II was expressed by tumor cells in only 5 of 21 tumors examined [167]. However, IGF-II mRNA expression was detected in nontumor tissue of all such tumors, including the adrenal cortex, stroma, capsule, and invading

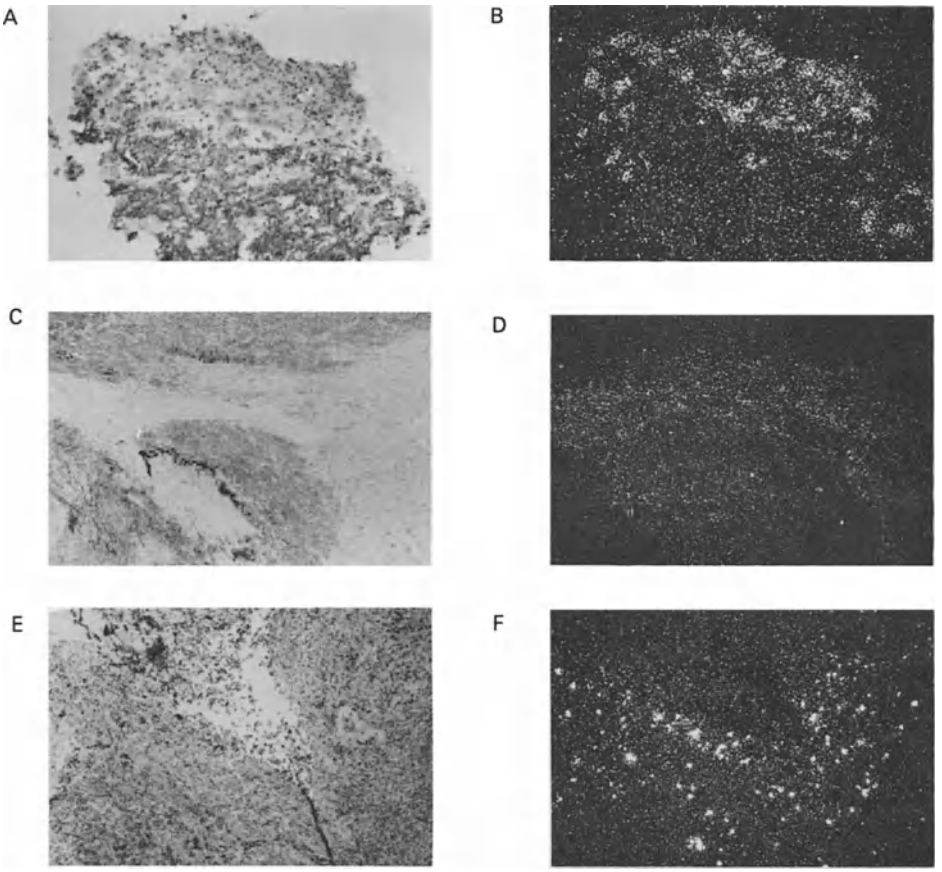

Figure 3. Expression of IGF-II in nonmalignant tissues associated with neuroblastoma tumors. Human neuroblastoma tumors were evaluated for IGF-II gene expression as described in the legend to Figure 2. Panels B, D, and F are dark field images of the same views shown in panels A, C, and E. Panels A and B demonstrate IGF-II gene expression in adrenal cortical cells surrounding an IGF-II negative neuroblastoma. Panels C and D demonstrate IGF-II gene expression in stromal tissue infiltrating an IGF-II negative neuroblastoma. Panels E and F demonstrate IGF-II gene expression in eosinophils associated with neuroblastoma tumor cells. (Panels C–F were reproduced with permission [167].)

eosinophils [167] (Fig. 3). Interestingly, we have observed that there may be an association between the number of eosinophils in neuroblastoma tumors and patient age and survival (Fig. 4). Although several reports have indicated that some hematopoietic cells may synthesize IGF-I [197–199], IGF-I or IGF-II gene expression in eosinophils or eosinophil infiltration of neuroblastomas has not been previously noted. If such cells do synthesize biologically active IGF-II, eosinophils associated with neuroblastoma tumors

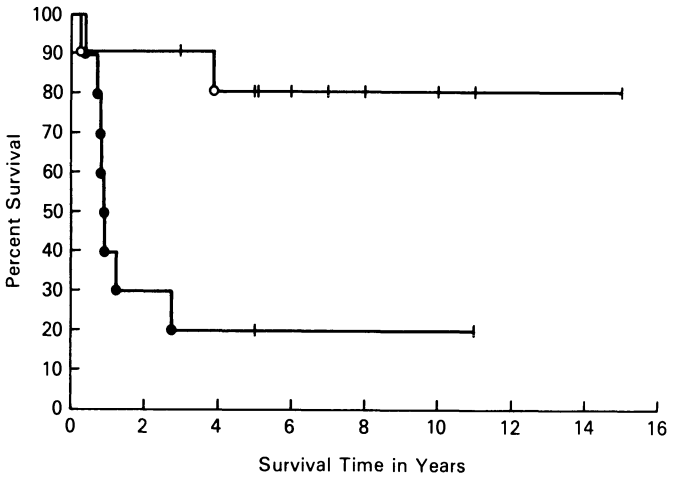

Figure 4. Association between number of eosinophils and survival in patients with neuroblastoma. Eosinophils were counted in paraffin sections of each of 21 tumors from patients presenting with neuroblastoma. To quantitate eosinophils in neuroblastoma tumor specimens, we first scanned nneuroblastoma tumor sections at 40× magnification using dark-field optics to identify 10 areas of the specimen that conntained the hiighest number of eosinophils. Using 200× magnification, we counted all of the eosinophils in each of 10 randomly chosen nonoverlapping fields within each of these areas. In tissues where eosinophils were rare, such that 10 fields containing eosinophils could not be readily identified using 40× magnification, the entire tissue was scanned at 200× magnification, and eosinophils in the 10 fields containing the greatest number of eosinophils were counted. Kaplan-Meier actuarial curves [205] demonstrate the relationship between eosinophil number (1–30, open circles; 30, closed circles) and survival. Mantel-Haenszel analysis of the data reporting 21 patients [206] yielded a two-tailed p value of 0.008.

may represent an important source of growth factor that can mediate neuroblastoma tumor cell proliferation.

Significance of IGF-II gene expression to metastasis and regression of neuroblastomas

In addition to stimulating the growth of human neuroblastoma tumor cells by autocrine or paracrine mechanisms, IGF-II gene expression may influence the sites at which metastatic neuroblastoma arises. Neuroblastomas typically metastasize to lymph nodes, bones, liver, or skin [1]. All of these tissues have been shown to express high levels of IGF-II mRNA or protein at the times during development when neuroblastomas are thought to arise [167, 181,189,190,195,200,201]. Also, IGF-II has been shown to be a chemoattractant for several human tumors [157,202]. These findings raise the possibility that neuroblastomas may metastasize to specific tissues due to the migratory influences of IGF-II. After invasion, the survival of neuro-

blastomas may be dependent on the availability of IGF-II in the microenvironment of the invaded tissue [167,169].

Neuroblastomas regress at a rate higher than that of any other human tumor. Tumor regression has been observed in neuroblastoma tissue located in the adrenal gland, liver, and skin [5,6]. Such regression may be due to the normal, although delayed, developmental decline of IGF-II production by such tissues. IGF-II expression in these tissues has been shown to decline from high levels observed early during gestation to low levels noted before birth or shortly after birth [167,181,190–192]. In our analysis, all four neuroblastomas in which IGF-II gene expression was detected in adrenal cortical cells adjacent to tumor were derived from patients newborn to 11 months of age. The tumors in three of these patients, newborn to 2 months of age, have regressed and these individuals are alive with no evidence of disease after 5–15 years of follow-up. The fourth patient died 5 months after diagnosis. Tumor tissue from this patient, aged 11 months, expressed IGF-II and contained numerous IGF-II positive eosinophils [167].

Concluding remarks

Several lines of investigation strongly support the view that neuroblastomas represent outgrowths of cells arrested at different stages during development [142,203]. Expression of N-*myc*, *ret*, *src*, and IGF-II, as well as the lack of expression of HLA in neuroblastoma cells, may indicate the time during development when such cells undergo malignant transformation. Cells blocked at different stages during the process of maturation are likely to have disparate growth potentials. An understanding of the different growth-regulating mechanisms operative during development may make it possible to design therapies that are appropriate for neuroblastoma tumors with distinctive biologic features.

IGF-II expression in neuroblastoma tumor cells and adjacent normal tissues may be important for the pathogenesis of neuroblastoma. IGF-II is not likely to initiate malignant transformation. Rather, IGF-II may mediate the autocrine or paracrine growth of neuroblastomas. Interestingly, neuroblastoma cell lines that express mRNA encoding IGF-II also express markers that identify mature adrenal medullary cells. Cell lines that do not express IGF-II express markers associated with less mature adrenal neuroblasts [167]. Lack of expression of IGF-II by neuroblastoma cells may indicate that such cells were transformed at a time during development when they did not express IGF-II. Such cells may require an exogenous source of IGF-II for growth. IGF-II gene expression by neuroblastoma tumor cells that arose from cells that physiologically express this gene may exhibit an autocrine growth mechanism utilizing endogenously produced IGF-II, as demonstrated for SK-N-AS cells [166].

The association of N-*myc* amplification and expression with the patho-

genesis of neuroblastomas remains a mystery. Clearly N-*myc* amplification is associated with a very poor prognosis, independent of age or stage. However, N-*myc* is amplified and overexpressed in only a minority of advanced-stage neuroblastomas [90,204]. For tumors in which N-*myc* is amplified or overexpressed, several questions remain. How does N-*myc* contribute to cellular transformation? Is N-*myc* a gene that induces autocrine growth factor production or metastasis? What are the pathologic characteristics of tumor progression with which N-*myc* amplification seems to correlate?

Nearly 60% of advanced-stage neuroblastomas lack detectable N-*myc* amplification at diagnosis [90] or at recurrence [204], yet in over 90% of such patients progressive disease develops [97]. This observation indicates that it will be important to identify and characterize other factors involved in the pathogenesis of neuroblastoma.

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References

1. Pizzo PA, Miser JS, Cassady JR, Filler RM: Solid tumors of childhood. In: Cancer: Principles and Practices of Oncology 2. DeVita VT, Jr., Hellman S, Rosenberg SA (eds): JB Lipincott, Philadelphia, 1985, pp 1511–1574.
2. Triche TJ, Cavazzana AO: Pathology in pediatric oncology. In: Pizzo PA, Poplack DG (eds): Principles and Practices of Pediatric Oncology. JB Lippincott, Philadelphia, 1989, pp 93–125.
3. Knudson AG Jr., Strong LC: Mutation and cancer: Neuroblastoma and pheochromocytoma. Am J Human Genet 24:514–532, 1972.
4. Israel MA: The evolution of clinical molecular genetics: Neuroblastoma as a model tumor. Am J Pediatr Hematol Oncol 8:163–172, 1986.
5. Evans AE, Gerson J, Schnaufer L: Spontaneous regression of neuroblastoma. Natl Cancer Inst Monogr 44:49–54, 1976.
6. Everson TC, Cole WH: Spontaneous regression of cancer. WB Saunders, Philadelphia, 1966, pp 11–87.
7. Knudson AG Jr., Meadows AT: Regression of neuroblastoma IV-S: A genetic hypothesis. N Engl J Med 302:1254–1256, 1980.
8. Brodeur GM, Seeger RC, Barrett A, et al.: International criteria for diagnosis, staging, and response to treatment in patients with neuroblastoma. J Clin Oncol 6:1874–1881, 1988.
9. Whang-Peng J, Triche TJ, Knutsen T, et al.: Cytogenetic characterization of selected small round cell tumors of childhood. Cancer Genet Cytogenet 21:185–208, 1986.
10. Triche TJ: Neuroblastoma and other childhood neural tumors: A review. Pediatr Pathol 10:175–193, 1990.

11. Ikeda I, Ishizaka Y, Tahira T, et al.: Specific expression of the ret proto-oncogene in human neuroblastoma cell lines. Oncogene 5:1291–1296, 1990.
12. Nagao M, Ishizaka Y, Nakagawara A, et al.: Expression of ret proto-oncogene in human neuroblastomas. Jpn J Cancer Res 81:309–312, 1990.
13. Vecchio G, Cavazzana AO, Triche TJ, et al.: Expression of the dbl proto-oncogene in Ewing's sarcomas. Oncogene 4:897–900, 1989.
14. Cox D, Yuncken C, Spriggs A: Minute chromatin bodies in malignant tumours of childhood. Lancet 2:55, 1965.
15. Biedler JL, Helson L, Spengler BA: Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. Cancer Res 33:2643–2652, 1973.
16. Biedler JL, Ross RA, Shanske S, et al.: Human neuroblastoma cytogenetics: Search for significance of homogeneously staining regions and double minute chromosomes. Prog Clin Biol Res 12:81–96, 1980.
17. Biedler JL, Meyers MB, Spengler BA: Homogeneously staining regions and double minute chromosomes: Prevalent cytogenetic abnormalities of human neuroblastoma cells. Adv Cell Neurobiol 4:267–307, 1983.
18. Brodeur GM, Sekhon GS, Goldstein MN: Chromosomal aberrations in human neuroblastomas. Cancer 40:2256–2263, 1977.
19. Brodeur GM, Green AA, Hayes FA, et al.: Cytogenetic features of human neuroblastomas and cell lines. Cancer Res 41:4678–4686, 1981.
20. Gilbert F, Feder M, Balaban G, et al.: Human neuroblastoma and abnormalities of chromosomes 1 and 17. Cancer Res 44:5444–5449, 1984.
21. Montgomery KT, Biedler JL, Spengler BA, et al.: Specific DNA sequence amplification in human neuroblastoma cells. Proc Natl Acad Sci USA 80:5724–5728, 1983.
22. Franke F, Rudolph B, Christiansen H, et al.: Tumour karyotype may be important in the prognosis of human neuroblastoma. J Cancer Res Clin Oncol 111:266–272, 1986.
23. Christiansen H, Lampert F: Tumour karyotype discriminates between good and bad prognostic outcome in neuroblastoma. Br J Cancer 57:121–126, 1988.
24. Biedler JL, Spengler BA: Metaphase chromosome anomaly: Association with drug resistance and cell-specific products. Science 191:185–187, 1976.
25. Alitalo K, Schwab M, Lin CC, et al.: Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-myc) in malignant neuroendocrine cells from a human colon carcinoma. Proc Natl Acad Sci USA 80:1707–1711, 1983.
26. Little CD, Nau MM, Carney DN, et al.: Amplification and expression of the c-myc oncogene in human lung cancer cell lines. Nature 306:194–196, 1983.
27. Gilbert F, Balaban G, Breg WR, et al.: Homogeneously staining region in a retinoblastoma cell line: Relevance to tumor initiation and progression. J Natl Caner Inst 67:301–306, 1981.
28. Kohl NE, Kanda N, Schreck RR, et al.: Transposition and amplification of oncogene-related sequences in human neuroblastoma. Cell 35:359–367, 1983.
29. Schwab M, Alitalo K, Klempnauer KH, et al.: Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 305:245–248, 1983.
30. Schwab M, Ellison J, Busch M, et al.: Enhanced expression of the human gene N-myc consequent to amplification of DNA may contribute to malignant progression of neuroblastoma. Proc Natl Acad Sci USA 81:4940–4944, 1984.
31. Brodeur GM, Seeger RC, Schwab M, et al.: Amplification of N-myc sequences in primary human neuroblastomas: Correlation with advanced disease stage. Prog Clin Biol Res 175:105–113, 1985.
32. Brodeur GM, Seeger RC: Gene amplification in human neuroblastomas: Basic mechanisms and clinical implications. Cancer Genet Cytogenet 19:101–111, 1986.
33. Shiloh Y, Korf B, Kohn NE, et al.: Amplifications and rearrangement of DNA sequences

from the chromosomal region 2p24 in human neuroblastoma. Cancer Res 46:5297–5301, 1986.
34. Suzuki T, Yokota J, Mugishima H, et al.: Frequent loss of heterozygosity on chromosome 14q in neuroblastoma. Cancer Res 49:1095–1098, 1989.
35. Srivatsan ES, Venugopal M, Seeger RC: Loss of heterozygosity for alleles on chromosomes 11q and 14q in neuroblastoma. Prog Clin Biol Res 366:91–98, 1991.
36. Gilbert F, Balaban G, Moorhead P, et al.: Abnormalities of chromosomes 1p in human neuroblastoma tumors and cell lines. Cancer Genet Cytogenet 7:33–42, 1982.
37. Schwab M: Is there a neuroblastoma anti-oncogene? Prog Clin Biol Res 366:1–10, 1991.
38. Mitlamn F: Catalog of Chromosome Aberrations in Cancer. Prog Top Cytogenet, 2nd ed., Vol. 5, Alan R. Liss, New York, 1985.
39. Hunt JD, Tereba AT: Molecular evaluation of abnormalities of the short arm of chromosome 1 in neuroblastoma. Genes Chromosomes Cancer 2:137–146, 1990.
40. Knudson AG Jr., Meadows AT: Developmental genetics of neuroblastoma. J Natl Cancer Inst 57:675–681, 1976.
41. Pasquale SR, Jones GR, Doersen C-J, et al.: Tumorigenicity and oncogene expression in pediatric cancers. Cancer Res 47:2715–2719, 1988.
42. Atkin NB: Chromosome 1 aberrations in cancer. Cancer Genet Cytogenet 21:279–285, 1986.
43. Fong CT, Dracopoli NC, White PS, et al.: Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: Correlation with N-myc amplification. Proc Natl Acad Sci USA 86:3753–3757, 1989.
44. Martinsson T, Weith A, Cziepluch C, et al.: Chromosome 1 deletions in human neuroblastomas: Generation and fine mapping of microclones from the distal 1p region. Genes Chromosomes Cancer 1:67–78, 1989.
45. Weith A, Martinsson T, Cziepluch C, et al.: Neuroblastoma consensus deletion maps to chromosome 1p36.1–2. Genes Chromosomes Cancer 1:159–166, 1989.
46. Look AT, Hayes FA, Nitschke R, et al.: Cellular DNA content as a predictor of response to chemotherapy in infants with unresectable neuroblastoma. N Engl J Med 311:231–235, 1984.
47. Hayashi Y, Hanada R, Yamamoto K, et al.: Chromosome findings and prognosis in neuroblastoma [letter]. Cancer Genet Cytogenet 29:175–177, 1987.
48. Hayashi Y, Inaba T, Hanada R, et al.: Chromosome findings and prognosis in 15 patients with neuroblastoma found by VMA mass screening. J Pediatr 112:567–571, 1988.
49. Hayashi Y, Kanda Ǹ, Inaba T, et al.: Cytogenetic findings and prognosis in neuroblastoma with emphasis on marker chromosome 1. Cancer 63:126–132, 1989.
50. Kaneko Y, Kanda N, Maseki N, et al.: Different karyotypic patterns in early and advanced stage neuroblastomas. Cancer Res 47:311–318, 1987.
51. Oppedal BR, Storm MI, Lie SO, et al.: Prognostic factors in neuroblastoma. Clinical, histopathologic, and immunohistochemical features and DNA ploidy in relation to prognosis. Cancer 62:772–780, 1988.
52. Bourhis J, DeVathaire F, Wilson GD, et al.: Combined analysis of DNA ploidy index and N-myc genomic content in neuroblastoma. Cancer Res 51:33–36, 1991.
53. Dominici C, Negroni A, Romeo A, et al.: Association of near-diploid DNA content and N-myc amplification in neuroblastomas. Clin Exp Metastasis 7:201–211, 1989.
54. Bishop JM: Molecular themes in oncogenesis. Cell 64:235–248, 1991.
55. Takahashi M, Cooper GM: ret transforming gene encodes a fusion protein homologous to tyrosine kinases. Mol Cell Biol 7:1378–1385, 1987.
56. Tahira T, Ishizaka Y, Itoh F, et al.: Characterization of ret proto-oncogene mRNAs encoding two isoforms of the protein product in a human neuroblastoma cell line. Oncogene 5:97–102, 1990.
57. Ishizaka Y, Ochiai M, Tahira T, et al.: Activation of the ret-II oncogene products differing in carboxy-termini due to alternative splicing. Oncogene 4:789–794, 1989.

58. Tahira T, Ishizaka Y, Sugimura T, et al.: Expression of proto-ret mRNA in embryonic and adult rat tissues. Biochem Biophys Res Commun 153:1290–1295, 1988.
59. Hunter T, Sefton BM: Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc Natl Acad Sci USA 77:1311–1315, 1980.
60. Collett MS, Purchio AF, Erikson RL.: Avian sarcoma virus-transforming protein, $pp60^{c-src}$ shows protein kinase activity for tyrosine. Nature 285:167–169, 1980.
61. Burgge JS, Cotton PC, Queral AE, et al.: Neurons express high levels of a structurally modified activated form of $pp60^{c-src}$. Nature 316:554–557, 1985.
62. Parsons SJ, Creutz CE.: $pp60^{c-src}$ activity detected in the chromaffin granule membrane. Biochem Biophys Res Commun 134:736–742, 1986.
63. Golden A, Nemeth SP, Brugge JS: Blood platelets express high levels of the $pp60^{c-src}$ specific tyrosine kinase activity. Proc Natl Acad Sci USA 83:852–856, 1986.
64. Mellstrom K, Bjelfman C, Hammerling U, et al.: Expression of c-src in cultured human neuroblastoma and small cell lung carcinoma cell lines correlates with neurocrine differentiation. Mol Cell Biol 7:4178–4184, 1987.
65. Bolen JB, Veillette A, Schwartz AM, et al.: Activation of $pp60^{c-src}$ protein kinase activity in human colon carcinoma. Proc Natl Acad Sci USA 84:2251–2255, 1987.
66. Cartwright CA, Kamps MP, Meisler AI, et al.: $pp60^{c-src}$ activation in human colon carcinoma. J Clin Invest 83:2025–2033, 1989.
67. Rosen N, Bolen JB, Schwartz AM, et al.: Analysis of $pp60^{c-src}$ protein kinase activity in human tumor cell lines and tissues. J Biol Chem 261:13754–13759, 1986.
68. Jocobs C, Rubsamen H: Expression of $pp60^{c-src}$ protein kinase in adult and fetal human tissue: High activities in some sarcomas and mammary carcinomas. Cancer Res 43:1696–1702, 1983.
69. Bolen JB, Rosen N, Israel MA: Increased $pp60^{c-src}$ tyrosyl kinase activity in human neuroblastomas is associated with amino-terminal tyrosine phosphorylation of the src gene product. Proc Natl Acad Sci USA 82:7275–7279, 1985.
70. O'Shaughnessy J, DeSeau V, Amini S, et al.: Analysis of the src gene product structure, abundance, and protein kinase activity in human neuroblastoma and glioblastoma cells. Oncogene Res 2:1–18, 1987.
71. Pyper JM, Bolen JP: Identification of a novel neuronal c-src exon expressed in human brain. Mol Cell Biol 10:2035–2040, 1990.
72. LeBeau JM, Wiestler OD, Walter G: An altered form of $pp60^{c-src}$ is expressed primarily in the central nervous system. Mol Cell Biol 7:4115–4117, 1987.
73. Bjelfman C, Hedborg F, Johansson I, et al.: Expression of the neuronal form of $pp60^{c-src}$ in neuroblastoma in relation to clinical stage and prognosis. Cancer Res 50:6908–6914, 1990.
74. Matsunaga T, Takahashi H, Ohnuma N, et al.: Expression of N-myc and c-src protooncogenes correlating to the undifferentiated phenotype and prognosis of primary neuroblastomas. Cancer Res 51:3148–3152, 1991.
75. Feramisco JR, Gross M, Kamata T, et al.: Microinjection of the oncogene form of the human Ha-ras (T-24) protein results in rapid proliferation of quiescent cells. Cell 38: 109–117, 1984.
76. Mulcahy LS, Smith MR, Stacy DW: Requirement for ras protooncogene function during serum stimulate growth of NIH3T3 cells. Nature 313:241–243, 1985.
77. Hurley JB, Simon MI, Teplow DB, et al.: Homologies between signal transducing G proteins and ras gene products. Science 226:860–862, 1984.
78. Birchmeier C, Broek D, Wigler M: Ras proteins can induce miosis in xenopus oocytes. Cell 43:615–621, 1985.
79. Tanaka T, Slamon DJ, Shimoda H, et al.: Expression of Ha-ras oncogene products in human neuroblastomas and the significant correlation with a patient's prognosis. Cancer Res 48:1030–1034, 1988.
80. Tanaka T: Tumor markers — personal experience. Ha-ras P21 in neuroblastoma: A new marker associating to patient's prognosis. Gan To Kagaku Ryoho 18:143–150, 1991.

81. Tanaka T, Ida N, Shimoda H, et al.: Organ specific expression of ras oncoproteins during growth and development of the rat. Mol Cell Biochem 70:97–104, 1986.
82. Noda M, Ko M, Ogura A, et al.: Sarcoma viruses carrying ras oncogenes induce differentiation-associated properties in neural cell line. Nature 318:73–75, 1985.
83. Bar-Sagi D, Feramisco JR: Microinjection of the ras oncogene protein into PC12 cells induce morphological differentiation. Cell 42:841–848, 1985.
84. Hagag N, Halegoua S, Viola M: Inhibition of growth factor-induced differentiation of PC12 cells by microinjection of antibody to ras p21. Nature 319:680–682, 1986.
85. Moley JF, Brother MB, Wells SA, et al.: Low frequency of ras gene mutations in neuroblastomas, pheochromocytomas, and medullary thyroid cancers. Cancer Res 51:1596–1599, 1991.
86. Dalla-Favera R, Wong-Staal F, Gallo RC: onc gene amplification in promyelocytic leukaemia cell line HL-60 and primary cells of the same patient. Nature 299:61–63, 1982.
87. Schwab M, Varmus HE, Bishop JM: Human N-myc contributes to neoplastic transformation of mammalian cells in culture. Nature 316:160–162, 1985.
88. Yancopoulos GD, Nisen PD, Tesfaye A, et al.: N-myc can cooperate with ras to transform normal cells in culture. Proc Natl Acad Sci USA 82:5455–5459, 1985.
89. Brodeur GM, Seeger RC, Schwab M, et al.: Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science 224:1121–1124, 1984.
90. Seeger RC, Brodeur GM, Sather H, et al.: Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med 313:1111–1116, 1985.
91. Nakagawara A, Ikeda K, Yokoyama T, et al.: Surgical aspects of N-myc amplification in relation to disease stage and histologic types in human neuroblastomas. Cancer 60: 820–826, 1987.
92. Tsuda T, Obara M, Hirano H, et al.: Analysis of N-myc amplification in relation to disease stage and histologic types in human neuroblastomas. Cancer 60:820–826, 1987.
93. Tsuda H, Shimosato Y, Upton MP, et al.: Retrospective study on amplification of N-myc and c-myc genes in pediatric solid tumors and its association with prognosis and tumor differentiation. Lab Invest 59:321–327, 1988.
94. Nakagawara A, Ikeda K, et al.: N-myc oncogene amplification and prognostic factors of neuroblastoma in children. J Pediatr Surg 22:895–898, 1987.
95. Nakagawara A, Ikeda K, Yokoyama T, et al.: Surgical aspects of N-myc oncogene amplification of neuroblastoma. Surgery 104:34–39, 1988.
96. Nakagawara A, Ikeda K: N-myc oncogene amplification and catecholamine metabolism in children with neuroblastoma. Lancet 1:559, 1987.
97. Phillips WS, Stafford PW, Duvol-Arnold B, et al.: Neuroblastoma and the clinical significance of N-myc oncogene amplification. Surg Gynecol Obstet 172:73–80, 1991.
98. Brodeur GM, Seeger RC, Sather H, et al.: Clinical implications of oncogene activation in human neuroblastomas. Cancer 58(Suppl. 2):541–545, 1986.
99. Tonini GP, Verdona G, DeBernardi B, et al.: N-myc oncogene amplification in a patient with IV-S neuroblastoma. Am J Pediatr Hematol Oncol 9:8–10, 1987.
100. Cohn SL, Herst CV, Maurer HS, et al.: N-myc amplification in an infant with stage IVs neuroblastoma. J Clin Oncol 5:1441–1444, 1987.
101. Carlsen NLT, Christensen IJ, Schroeder H, et al.: Prognostic value of different staging systems in neuroblastomas and completeness of tumour excision. Arch Dis Child 61: 832–842, 1986.
102. Garvin J Jr., Bendit I, Nisen PD: N-myc oncogene expression and amplification in metastatic lesions of stage IV-S neuroblastoma. Cancer 65:2572–2575, 1990.
103. Nakagawara A, Sasazuki T, Akiyama H, et al.: N-myc oncogene and stage IV-S neuroblastoma. Cancer 65:1960–1967, 1990.
104. Michitsch R, Biedler JL, Melera P: Modulation of N-myc expression, but not tumorigenicity accompanies phenotypic conversion of neuroblastoma cells in prolonged culture. Prog Clin Biol Res 271:103–120, 1988.

105. Slamon DJ, Boone TC, Seeger RC, et al.: Identification and characterization of the protein encoded by the human N-myc oncogene. Science 232:768–772, 1986.
106. Ramsey G, Stanton L, Schwab M, et al.: Human proto-oncogene N-myc encodes nuclear proteins that bind DNA. Mol Cell Biol 6:4450–4467, 1986.
107. Seeger RC, Wada R, Brodeur GM, et al.: Expression of N-myc by neuroblastomas with one or multiple copies of the oncogene. Prog Clin Biol Res 271:41–49, 1988.
108. Kohl NE, Gee CE, Alt FW: Activated expression of the N-myc gene in human neuroblastomas and related tumors. Science 226:1335–1337, 1984.
109. Schwab M, Varmus H, Bishop J, et al.: Chromosome localization in normal human cells and neuroblastomas of a gene related to c-myc. Nature 308:288–291, 1984.
110. Alt F, DePinho R, Zimmerman K, et al.: The human myc gene family. Cold Spring Harbor Symp Quant Biol 51:931–941, 1986.
111. Zimmerman K, Yancopoulos G, Collum R, et al.: Differential expression of myc family genes during murine development. Nature 319:780–783, 1986.
112. Hirvonen H, Sandberg M, Kalimo H, et al.: The N-myc proto-oncogene and IGF-II growth factor mRNAs are expressed by distinct cells in human fetal kidney and brain. J Cell Biol 108:1093–1104, 1989.
113. Grady EF, Schwab M, Rosenau W: Expression of N-myc and c-src during the development of fetal human brain. Cancer Res 47:2931–2936, 1987.
114. Dildrop R, Zimmerman KD, DePinho R, et al.: Differential expression of myc family genes during development: Normal and deregulated N-myc expression in transgenic mice. Curr Top Microbiol Immunol 141:100–109, 1988.
115. Dildrop R, Ma A, Zimmerman KD, et al.: IgH enhancer-mediated deregulation of N-myc gene expression in transgenic mice: Generation of lymphoid neoplasias that lack c-myc expression. EMBO J 8:1121–1128, 1989.
116. Dildrop R, Zimmerman KD, Yancopoulos G, et al.: Differential expression of myc-family genes during development: Normal and deregulated N-myc expression in transgenic mice. Curr Top Microbiol Immunol 51:931–941, 1986.
117. Lee WH, Murphree AC, Benedict WF: Expression and amplification of the NMYC gene in primary retinoblastoma. Nature 309:458–460, 1984.
118. Nau M, Brooks B, Carney D, et al.: Human small-cell lung cancers show amplification and expression of the N-myc gene. Proc Natl Acad Sci USA 83:1092–1096, 1986.
119. Rosolen A, Whitesell L, Ikegaki N, et al.: Antisense inhibition of single copy N-myc expression results in decreased cell growth without reduction of c-myc protein in a neuroepithelioma cell line. Cancer Res 50:6316–6322, 1990.
120. Schweigerer L, Breit S, Wenzel A, et al.: Augmented MYCN expression advances the malignant phenotype of human neuroblastoma cells: Evidence for induction of autocrine growth factor activity. Cancer Res 50:4411–4416, 1990.
121. Rosen N, Reynolds C, Thiele C, et al.: Increased N-myc expression following progressive growth of human neuroblastoma. Cancer Res 46:4139–4142, 1986.
122. Berman SA, Bursztajn S, Kinnard R, et al.: Increased N-MYC mRNA expression associated with dibutyryl cyclic AMP induced neuroblastoma differentiation. J Neurogenet 6:75–86, 1989.
123. Thiele CJ, Israel MA: Regulation of N-myc expression is a critical event controlling the ability of human neuroblasts to differentiate. Exp Cell Biol 56:321–333, 1988.
124. Zimmerman K, Alt FW: Expression and function of myc family genes. Crit Rev Oncog 2:75–95, 1990.
125. Thiele CJ, Reynolds CP, Israel MA: Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. Nature 313:404–406, 1985.
126. Amatruda TT, III, Sidell N, Ranyard J, et al.: Retinoic acid treatment of human neuroblastoma cells is associated with decreased N-myc expression. Biochem Biophys Res Commun 126:1189–1195, 1985.
127. Horii Y, Sugimoto T, Swada T, et al.: Differential expression of N-myc and c-src

protooncogenes during neuronal and schwannian differentiation of human neuroblastoma cells. Int J Cancer 43:305–309, 1989.
128. Cornaglia-Ferraris P, Tonini GP: Letter to the editor. Am J Pediatr Hematol Oncol 10:182–183, 1988.
129. Festinstein H, Schmidt W: Variation in MHC antigenic profiles of tumor cells and its biologic effects. Immunol Rev 60:85–127, 1981.
130. Katzav S, de Baetselier P, Tartakowsky B, et al.: Alterations in major histocompatibility complex phenotypes of mouse cloned T10 sarcoma cells; association with shifts from nonmetastatic to metastatic cells. J Natl Cancer Inst 71:317–324, 1983.
131. Schmidt W, Atfield G, Festenstein H: Loss of H-2 gene products from AKR spontaneous leukemias. Immunogenetics 8:311–, 1979.
132. Schrier PI, Bernards R, Vaessen MJ, et al.: Expression of class I major histocompatibility antigen switched off by highly oncogenic adenovirus 12 in transformed rat cells. Nature 305:771–775, 1983.
133. Hui K, Grosveld F, Festinstein H: Rejection of transplantable AKR leukemia cells following MHC DNA-mediated cell transformation. Nature 311:750–752, 1984.
134. Tanaka K, Isselbacher K, Khoury G, et al.: Reversal of oncogenesis by the expression of a major histocompatibility complex class I gene. Science 228:26–30, 1985.
135. Wallich R, Bulbue M, Hammerling G, et al.: Albrogation of metastatic properties of tumor cells by '*de novo*' expression of H-2K antigens following H-2 gene transfection. Nature 315:301–305, 1985.
136. Salerno C, Crepaldi T, Savoia P, et al.: Expression of HLA class I antigens in human tumors and their involvement in tumor growth. Res Clin Lab 20:85–93, 1990.
137. Lampson LA, Fisher CA, Whelan JP: Striking paucity of HLA-A, B, C and *B*2-microglobulin on human neuroblastoma cell lines. J Immunol 130:2471–2478, 1983.
138. Favrot MC, Combaret V, Goillot E, et al.: Expression of leucocyte adhesion molecules on 66 clinical neuroblastoma specimens. Int J Cancer 48:502–510, 1991.
139. Dausset J: The major histocompatibility complex in man. Science 213:1469–1474, 1981.
140. Main EK, Lampson LA, Hart MK, et al.: Human neuroblastoma cells are susceptible to lysis of natural killer cells but not by cytotoxic T lymphocytes. J Immunol 135:242–246, 1985.
141. Lampson LA: Biological significance of HLA-A, B, C expression in neuroblastoma and related cell lines. Prog Clin Biol Res 271:409–420, 1988.
142. Cooper MJ, Huchins GM, Cohen PS, et al.: Human neuroblastoma tumors correspond to the arrested differentiation of chromaffin adrenal medullary neuroblasts. Cell Growth Differ 1:149–159, 1990.
143. Lampson LA, Fisher CA: Weak HLA and beta 2-microglobulin expression of neuronal cell lines can be modulated by interferon. Proc Natl Acad Sci USA 81:6476–6480, 1984.
144. Houghton AN, Thomson TM, Gross D, et al.: Surface antigens of melanoma and melanocytes. Specificity of induction of Ia antigens by human gamma-interferon. J Exp Med 160:255–269, 1984.
145. Gross N, Beck D, Favre S, et al.: In vitro antigenic modulation of human neuroblastoma cells induced by IFN-gamma, retinoic acid and dibutyryl cyclic AMP. Int J Cancer 39:521–529, 1987.
146. Sugimoto T, Horii Y, Hino T, et al.: Differential susceptibility of HLA class II antigens induced by gamma-interferon in human neuroblastoma cell lines. Cancer Res 49: 1824–1828, 1989.
147. Gross N, Beck D, Favre S: In vitro modulation and relationship between N-myc and HLA class I RNA steady-state levels in human neuroblastoma cells. Cancer Res 50:7532–7536, 1990.
148. Evans A, Main E, Zier K, et al.: The effects of gamma interferon on the natural killer and tumor cells of children with neuroblastoma. Cancer 64:1383–1387, 1989.
149. Reynolds CP, Tomayko MM, Donner L, et al.: Biological classification of cell lines derived from human extra-cranial neural tumors. Prog Clin Biol Res 271:291–306, 1988.

150. Bernards R, Dessain SK, Weinberg RA: N-myc amplification causes down-modulation of MHC class I antigen expression in neuroblastoma. Cell 47:667–674, 1986.
151. Versteeg R, Noordermeer I, Kruse-Wolters M, et al.: c-myc down-regulates class I HLA expression in human melanomas. EMBO J 7:1023–1029, 1988.
152. Versteeg R, van der Minne C, Plomp A, et al.: N-myc expression switched off and class I human leukocyte antigen expression switched on after somatic cell fusion of neuroblastoma cells. Molec Cell Biol 10:5416–5423, 1990.
153. Feltner DE, Cooper M, Weber J, et al.: Expression of class-I histocompatibility antigens in neuro-ectodermal tumors is independent of the expression of a transfected neuroblastoma myc gene. J Immunol 143:4292–4299, 1989.
154. Sporn MB, Roberts AB: Autocrine growth factors and cancer. Nature 313:745–747, 1985.
155. Cuttitta F, Carney DN, Mulshine J, et al.: Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. Nature 316:823–826, 1985.
156. Schwab G, Siegall CB, Aarden LA, et al.: Characterization of an interlukin-6-mediated autocrine growth loop in the human multiple myeloma cell line, U266. Blood 77:587–593, 1991.
157. El-Badry OM, Minniti C, Kohn EC, et al.: Insulin-like growth factor II acts as an autocrine growth and motility factor in human rhabdomyosarcoma tumors. Cell Growth Differ 1:325–331, 1990.
158. Yee D, Cullen KJ, Paik S, et al.: Insulin-like growth factor II mRNA expression in human breast cancer. Cancer Res 48:6691–6696, 1988.
159. Yee D, Paik S, Lebovic GS, et al.: Analysis of insulin-like growth factor I gene expression in malignancy: Evidence for a paracrine role in human breast cancer. Mol Endocrinol 3:509–517, 1989.
160. Ross RA, Spengler BA, Biedler JL: Coordinate morphological and biochemical interconversion of human neuroblastoma cells. J Natl Cancer Inst 71:741–747, 1983.
161. Ciccarone V, Spengler B, Meyers M, et al.: Phenotypic diversification in human neuroblastoma cells: Expression of distinct neural crest lineages. Cancer Res 49:219–225, 1989.
162. Rettig W, Spengler B, Chesa P, et al.: Coordinate changes in neuronal phenotype and surface antigen expression in human neuroblastoma cell variants. Cancer Res 47: 1383–1389, 1987.
163. Bossart E, Conti G: Epidermal growth factor stimulates colony formation and non-neuronal marker protein expression by human neuroblastoma in methylcellulose culture. Anticancer Res 9:1497–1504, 1989.
164. Suardet L, Gross N, Gaide A-C, et al.: Epidermal growth factor responsiveness of a new human neuroblastoma cell line. Int J Cancer 44:661–668, 1989.
165. Ludecke G, Unsicker K: Mitogenic effects of neurotrophic factors on human neuroblastoma IMR 32 cells. Cancer 65:2270–2277, 1990.
166. El-Badry OM, Romanus JA, Helman LJ, et al.: Autonomous growth of a human neuroblastoma cell line is mediated by insulin-like growth factor II. J Clin Invest 84: 829–839, 1989.
167. El-Badry OM, Helman LJ, Chatten J, et al.: Insulin-like growth factor II-mediated proliferation of human neuroblastoma. J Clin Invest 87:648–657, 1991.
168. El-Badry OM, Meyers MB, Spengler BA, et al.: Medium conditioned by human neuroblastoma BE(2)-C cells contains an autocrine/paracrine acting growth factor with properties similar to insulin-like growth factor II. Prog Clin Biol Res 366:257–266, 1991.
169. El-Badry OM: Insulin-like growth factor II gene expression in human neuroblastoma. Prog Clin Biol Res 366:249–256, 1991.
170. Van Wyk JJ, Graves DC, Casella SJ, et al.: Evidence from monoclonal antibody studies that insulin stimulated deoxyribonucleic acid synthesis through the type I somatomedin receptor. J Clin Endocrinol Metab 61:639–643, 1985.
171. Flier SJ, Usher P, Moses AC: Monoclonal antibody to the type I insulin-like growth factor

(IGF-I) receptor blocks IGF-I and insulin in human skin fibroblasts. Proc Natl Acad Sci USA 83:664–668, 1986.
172. Furlanetto RW, DiCarlo JN, Wisehart C: The type II insulin-like growth factor receptor does not mediate deoxyribonucleic acid synthesis in human fibroblasts. J Clin Endocrinol Metab 64:1142–1149, 1987.
173. Ota AW, Wilson GL, Spilberg O, et al.: Functional insulin-like growth factor I (IGF-I) receptors are expressed by neural derived continuous cell lines. Endocrinology 122:145–152, 1988.
174. Ota AW, Wilson GL, LeRoith D: Insulin-like growth factor I receptors on mouse neuroblastoma cells: Two B subunits are derived from differences in glycosylation. Eur J Biochem 174:521–530, 1988.
175. Sturm MA, Conover CA, Pham H, et al.: Insulin-like growth factors and receptors and binding protein in rat neuroblastoma cells. Endocrinology 124:388–396, 1989.
176. Mattsson MEK, Hammerling U, Mohall E, et al.: Mitogenically uncoupled insulin and IGF-I receptors of differentiated human neuroblastoma cells are functional and mediate ligand-induced signals. Growth Factors 2:251–265, 1990.
177. Chelmicka-Schorr E, Checinski ME, Arnason BGW: PC12 pheochromocytoma and sympathetic nervous system derived trophic factors augment growth of neuroblastoma. Eur J Cancer Clin Oncol 25:1057–1059, 1989.
178. Chelmicka-Schorr E, Checinski ME: Sympathetic nervous system derived trophic factor augments growth of human neuroblastoma in vitro. Eur J Cancer Clin Oncol 25:393–394, 1989.
179. Brown AL, Graham EE, Nissley SP, et al.: Developmental regulation of insulin-like growth factor II mRNA in different rat tissues. J Biol Chem 261:13144–13150, 1986.
180. Levinovitz A, Norstedt G: Developmental and steroid hormonal regulation of insulin-like growth factor II expression. Mol Endocrinol 3:797–804, 1989.
181. Scott J, Cowell JJ, Robertson ME, et al.: Insulin-like growth factor-II gene expression in Wilms' tumor and embryonic tissues. Nature 317:260–262, 1985.
182. van Dijk JP, Tanswell AK, Challis JRG: Insulin-like growth factor (IGF)-II and insulin, but not IGF-I are mitogenic for fetal rat adrenal cells in vitro. J Endocrinol 119:509–516, 1988.
183. Froesch ER, Shmid C, Schwander J, et al.: Actions of insulin-like growth factors. Annu Rev Physiol 47:443–467, 1985.
184. Sara VR, Hall K, Misaki M, et al.: Ontogenesis of somatomedin and insulin receptors in the human fetus. J Clin Invest 71:1084–1094, 1983.
185. Shigematsu KM, Niwa M, Kurihara M, et al.: Receptor autoradiographic localization of insulin-like growth factor-I (IGF-I) binding sites in human fetal and adult adrenal glands. Life Sci 45:383–389, 1989.
186. Pillion DJ, Yang M, Grizzle WE: Distribution of receptors for insulin and insulin-like growth factor I (Somatomedin C) in the adrenal gland. Biochem Biophys Res Commun 154:138–145, 1988.
187. Furlanetto RW, Underwood LE, Van Wyk JJ, et al.: Estimation of somatomedin-C levels in normals and patients with pituitary disease by radioimmunoassay. J Clin Invest 60: 648–657, 1977.
188. Schoenle E, Zapf J, Humbel RE, et al.: Insulin-like growth factor I stimulates growth in hypophysectomized rats. Nature 296:252–253, 1982.
189. Han VKM, D'Ercole AJ, Lund PK: Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. Science 236:193–197, 1987.
190. Gray A, Tam AW, Dull TJ, et al.: Tissue-specific and developmentally regulated transcription of the insulin-like growth factor 2 gene. DNA 6:283–295, 1987.
191. Voutilainen R, Miller WL: Developmental and hormonal regulation of mRNAs for insulin-like growth factor II and steroidogenic enzymes in human fetal adrenals and gonads. DNA 7:9–15, 1988.

192. Brice AL, Cheetham JE, Bolton VN, et al.: Temporal changes in the expression of the insulin-like growth factor II gene associated with tissue maturation in the human fetus. Development 106:543–554, 1989.
193. Crowder RE: The development of the adrenal gland in man, with special reference to the origin and ultimate location of cell types and evidence in favour of the 'cell migration theory.' Contrib Embryol 36:195–210, 1957.
194. Turkel SB, Itabashi HH: The natural history of neuroblastic cells in the fetal adrenal gland. Am J Pathol 76:225–244, 1974.
195. Han VKM, Lund PK, Lee DC: Expression of somatomedin/insulin-like growth factor messenger ribonucleic acids in the human fetus: Identification, characterization, and tissue distribution. Clin Endocrinol Metab 66:422–429, 1988.
196. Suzuki T, Iwafuchi M, Yanaihara C, et al.: IGF-II-like immunoreactivity in human tissues, neuroendocrine tumors, and PC12 cells. Diabetes Res Clin Pract 7:S21–S27, 1989.
197. Rom WN, Basset P, Fells GA, et al.: Alveolar macrophages release an insulin-like growth factor I-type molecule. J Clin Invest 82:1685–1693, 1988.
198. Karey KP, Sirbasku DA: Human platelet-derived mitogens. II. Subcellular localization of insulinlike growth factor I to the alpha-granule and release in response to thrombin. Blood 74:1093–1100, 1989.
199. Merimee TJ, Grant MB, Broder CM, et al.: Insulin-like growth factor secretion by human B-lymphocytes: A comparison of cells from normal and pygmy subjects. J Clin Endocrinol Metab 69:978–984, 1989.
200. Mohan S, Jennings JC, Linkhart TA, et al.: Primary structure of human skeletal growth factor: Homology with human insulin-like growth factor-II. Biochim Biophys Acta 966: 44–55, 1988.
201. Frolik CA, Ellis LF, Williams DC: Isolation and characterization of insulin-like growth factor-II from human bone. Biochem Biophys Res Commun 151:1011–1018, 1988.
202. Stracke ML, Engel JD, Wilson LW, et al.: The type I IGF receptor is a motility receptor in human melanoma cells. J Biol Chem 264:21544–21549, 1989.
203. Thiele CJ, McKeon C, Triche TJ, et al.: Differential protooncogene expression characterizes histopathologically indistinguishable tumors of the peripheral nervous system. J Clin Invest 80:804–811, 1987.
204. Brodeur GM, Hayes FA, Green AA, et al.: Consistent N-myc copy number in simultaneous or consecutive neuroblastoma samples from sixty individual patients. Cancer Res 47:4248–4253, 1987.
205. Kaplan E, Meier P: Non-parametric estimation from incomplete observations. J Am Stat Assoc 53:457–481, 1958.
206. Mentel N: Evaluation of survival data and two new rank order statistics arising in its consideration. Canc Chem Rep 50:163–170, 1966.

6. Kaposi sarcoma: A cytokine-responsive neoplasia?

Steven A. Miles

Introduction

Kaposi sarcoma is the most common malignant complication of HIV infection [1,2]. While the incidence has steadily declined since AIDS was first described [3–5], Kaposi sarcoma still affects nearly 30% of patients at some time during their disease course. Since both the extent of disease and rate of progression varies widely between patients [6], cytokines are hypothesized to be involved in the pathogenesis of this neoplasia [7–11].

Kaposi sarcoma is also seen in endemic and indolent forms [12,13]. The former occurs in African males and the later primarily in men of mediterranean descent. The precise reasons for the male predilection of both classical and epidemic forms, and the marked increased incidence of Kaposi sarcoma in patients with HIV infection, are unknown. However, a careful evaluation of the epidemiology and clinical presentation of this disorder reveals some clues to its pathogenesis. Recent laboratory studies provide support to some of these hypotheses and may provide new avenues of therapeutic intervention. Completed studies suggest that both angiogenic growth factors and an altered progenitor cell are keys to the development of Kaposi sarcoma [8,14–17].

Epidemiology of Kaposi sarcoma

There are several unique characteristics of Kaposi sarcoma that must be explained by any model for its pathogenesis. In the case of HIV-associated Kaposi sarcoma, these include a high male to female ratio in the United States [1,2,6,18,19], the preponderance of disease in patients with sexually transmitted HIV infection [12,20–27], its declining incidence in the U.S. population of HIV patients [5,28], and its appearance in individuals at risk for HIV infection but without detectable HIV infection [19,29–33]. Clinically, the neoplasia can be indolent or aggressive and frequently has exacerbations at the time of opportunistic infections. This stepwise progression of disease suggests the presence of circulating growth factor(s) [7–9,14,34].

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To a certain extent, these features are also seen in endemic and classical Kaposi sarcoma, but there are some exceptions. For example, the preponderance of affected individuals are male and most have subtle immunologic abnormalities [12,13]. Affected patients have an increased incidence of low-grade lymphomas. Similar to HIV patients, the incidence of Kaposi sarcoma appears to be increased in cytomegalovirus-infected patients [35–39]. However, nearly all lack evidence of HIV infection as well as infection with other known human retroviruses.

Kaposi sarcoma also occurs occassionally in patients who have received organ transplants and who are iatrogenically immunosuppressed. Interestingly, these patients are usually receiving steroids and have been exposed to cytomegalovirus. After withdrawal of the immunosuppression, the Kaposi sarcoma lesions typically regress [40].

Together, the epidemiology of all three forms of disease suggests that Kaposi sarcoma is associated with some form of immunosuppression. Whether this association is causative (from defects in immunosurveillance) or simply an exacerbation of a premalignant lesion from altered cytokine production is unknown. Growth of Kaposi sarcoma can be modified by both glucocorticoids and perhaps the sex steroids. This may potentially explain the male predonderance. More controversial is whether Kaposi sarcoma could be the result of cytomegalovirus infection or another sexually transmitted agent [29,41–43] or HIV [14,16]. Its regression with immune modification and alpha interferon [44–49] suggests that Kaposi sarcoma maybe a neoplasia that is driven by circulating mitogens [50–52].

In vitro studies of Kaposi sarcoma cells

Kaposi sarcoma derived cells (KS cells) were first grown in long-term culture with conditioned media from retrovirally infected T-cell lines [8]. Although the cells were isolated from lung tissue, careful histologic examination of the isolated cells confirmed earlier immunohistochemical studies of Kaposi sarcoma skin lesions [8]. The isolated cells typically stain with *Ulex Europeas I* lectin, acetylated LDL receptors, in some cases vimentin, and variably for Von Willebrand's factor. Subsequent studies detected both desmin and smooth muscle alpha actin, suggesting that the cultured cells are mesenchymal progenitor cells that possess both endothelial and smooth muscle antigens [53]. Since the cells are isolated in a complex media with multiple cytokines, it is important to note that the expression of some of these antigens may be artifactual and the result of current isolation and growth processes [53].

Oncogenic potential of Kaposi sarcoma DNA

Southern analysis of DNA from Kaposi sarcoma lesions [37,38] and derived cells [8] fails to detect any sequences that are homologous to HIV or other

human retroviruses. Evidence of cytomegalovirus infection is typically found. However, given the very high rate of CMV infection in patients with HIV, it is likely that CMV may represent a comorbid factor rather than an etiologic agent. In other studies, transfection of DNA from human Kaposi sarcoma lesions identified the presence of an oncogene called ks-FGF [54–56]. This member of the fibroblast growth factor family is related to the *hst* (int) oncogene. The gene product ks-FGF uses bFGF receptors, is homologous with bFGF, and acts as a mitogen for fibroblasts, endothelial cells, and melanocytes. More detailed studies of skin lesions show that this gene is more important in other epithelial tumors than in epidemic Kaposi sarcoma.

Additional transforming sequences in AIDS-KS and classical KS DNA have been described [57–59]. Using transfection of NIH3T3 cells with DNA extracted from a KS tissue, several primary transformants, containing human repetitive DNA, were identified and shown to be anchorage independent and tumorigenic in nude mice. This occurs approximately 5–10 times higher than background transfectants. DNA extracted from such clones was transfected in NIH3T3 cells for a second round of transfection. A single fragment of DNA (5.4 kB) containing Alu homologous sequences was identified and is now being sequenced.

A separate group has also identified transforming sequences with a frequency of approximately 0.02 foci per 5×10^5 cells/μg DNA in NIH 3T3 cells [60]. The primary and secondary transfectants contain human repetitive DNA sequences. The transfected clones produce hemorrhagic angiosarcomatous neoplasms when implanted in nude mice with a histology that is very similar to human Kaposi sarcoma. The tumor produced by some transfectants is highly invasive and metastatic in nude mice. No significant homologues of *ras*N, *ras*H, *ras*K, v-*sis*, v-*src*, and v-*fes* oncogenes (known to transform NIH/3T3 cells) were identified in the Kaposi sarcoma DNA-transformed cells. Overall these studies indicate that there are acutely transforming sequences present in Kaposi sarcoma that are human and not of infectious origin. Unfortunately, without the identification of these sequences, it is not possible to tell whether they represent new oncogenes and where these findings fit into the process of transformation.

Angiogenic growth factors

Factors in the conditioned media from several human retrovirus-infected T cells promote the growth of Kaposi sarcoma cells and, to a lesser extent, normal human endothelial cells [8]. Moreover, both the media and protein extracts from the Kaposi sarcoma cells support the growth and proliferation of newly derived Kaposi sarcoma cells [8,61,62]. This suggests that both retrovirus-infected T cells and Kaposi sarcoma cells produce angiogenic factors that could increase the growth of Kaposi sarcoma. The angioblastic reaction observed at the site of inoculation of Kaposi sarcoma cells in

nude mice confirms that Kaposi sarcoma cells produce substances that are angiogenic [61].

Additional studies of potential angiogenic factors show that Kaposi sarcoma cells express basic fibroblast growth factor (bFGF), IL-1 alpha [61], and IL-6 [9]. Both classical and AIDS-associated Kaposi sarcoma cells also appear to be more responsive to PDGF than their normal counterparts [63–66]. This may give them a growth advantage. High-affinity receptors for several cytokines, including IL-1, IL-2, IL-6, IL-8, TNF, and PDGF, are present on Kaposi sarcoma cells [63,78]. These receptors may be functional as antibodies to IL-1, bFGF, or PDGF and antisense oligonucleotides to IL-6 inhibit Kaposi sarcoma cells [9,61]. Since Kaposi sarcoma cells make and respond to several of these cytokines [79], Kaposi sarcoma cells could participate in multiple paracrine and autocrine growth loops in vitro and in vivo.

Production of several of these factors by mesenchymal cells is normal and does not necessarily indicate that Kaposi sarcoma cells possess an altered cell phenotype. For example, expression of IL-6 by mesenchymal tissues is normal [67]. However, the unusual observation is that Kaposi sarcoma cells, but not endothelial or smooth muscle cells, respond to IL-6 [9,67]. This also appears to be the case with Oncostatin-M [79]. The unique proliferative response of these cells to these cytokines may distinguish Kaposi sarcoma cells from their normal mesenchymal counterparts. In addition, since both IL-6 and IL-6 receptor mRNA are found in AIDS Kaposi sarcoma lesions, this autocrine growth loop may be functional in vivo [9].

IL-6 may also be important because of its central role in modulating the response of these cells to other cytokines that are known to be perturbed in HIV-infected individuals. Because multiple cytokines can modulate IL-6 in vivo and in vitro [77], it is possible that increases in the level of other cytokines could increase IL-6 and thereby increase the growth of Kaposi sarcoma lesions. For example, both TNF-α and IL-1β are often increased in patients with HIV [68,82]. This expression is transient and usually occurs coincident with opportunistic infections. It is interesting to speculate that increases in one or several of these cytokines during opportunistic infections could increase IL-6 within Kaposi sarcoma cells and increase tumor cell growth. This could explain the explosive growth of tumors seen at times of opportunistic infections.

Role of the HIV in the development of Kaposi sarcoma

An additional factor, the transactivating protein of HIV, HIV-*tat*, may be a mitogen for Kaposi sarcoma cells [50,69]. HIV-*tat* increases the proliferation of Kaposi sarcoma cells but has no effect on smooth muscle or endothelial cell cultures. However, the mitogenic effects of HIV-*tat* are modest (less than twofold) and are not of the magnitude seen with cytokines such as

oncostatin-M, TNF-α, or IL-1β [77]. Nonetheless, the demonstration that HIV-*tat* could contribute to the development of AIDS-associated Kaposi sarcoma is important for several reasons. First, it confirms earlier animal data that HIV-*tat*, under the control of the HIV LTR, could produce Kaposi sarcoma-like lesions in male transgenic mice [70]. Despite a variety of attempts, multiple groups were unsuccessful in reproducing this finding. Second, it is the first demonstration that HIV could directly induce angiogenesis and may be the sole factor necessary for the development of HIV-associated Kaposi sarcoma. If true, it would obviate the need to postulate a role for CMV or other sexually transmitted agents [29,41–43]. Finally, it provides a rationale for the use of *tat* inhibitors as primary therapy for Kaposi sarcoma [71].

As attractive as this possibility is, there are several problems with the hypothesis and several limitations of the research. First, HIV-*tat* was found to have activity in *trans* at nanogram concentrations. Others groups working with a variety of *tat* preparations find that transcellular activation by recombinant *tat* is seen at microgram concentrations [72,73], raising doubt as to the specificity of the response. Further, it is postulated that HIV-*tat* enters the cells at this low concentration via an RGD receptor [69,74]. Preliminary reports suggest that IL-1, TNF-α, and retroviral supernatants can increase expression of RGD receptors on normal endothelial cells, facilitating *tat* activity [83]. Thus, according to this theory, cytokines could increase a *tat* receptor on mesenchymal cells. Once inside the cell, *tat* would act as a mitogen, giving the *tat*-containing cells a growth advantage. This is unlikely for several reasons.

While applicable to AIDS-associated Kaposi sarcoma, this theory ignores the obvious lack of a role for HIV-*tat* in the pathogenesis of classical Kaposi sarcoma. Also, not all patients with HIV infection, and hence HIV-*tat*, develop Kaposi sarcoma despite the profound immunosuppression seen in all patients. Second, these experiments are carried out in systems using both heparin and gelatin. The former is known to inhibit the activity of *tat* in transcription and the later is rich in RGD sequences. It is difficult to believe that *tat* at nanogram concentrations is capable of overcoming both these obstacles when other systems require *tat* concentrations at least two orders higher in the absense of heparin and gelatin. Several groups have also shown that the major route of uptake of *tat* is via the arginine-rich basic region of HIV-*tat*, not the RGD sequence found in the second exon. In fact, *tat* that is deficient in the second exon is quite capable of entering cells and transactivating HIV-LTR DNA constructs [73,75,76]. Thus, RGD-mediated specific uptake of HIV-*tat* is unlikely. Moreover, several attempts at identifying circulating *tat* in patients with HIV have failed, as well as attempts to find *tat* sequences in lesions or mononuclear cells of HIV-negative homosexual men with Kaposi sarcoma [77]. Since HIV retroviral sequences are not found in classical Kaposi sarcoma, the data suggest that *tat* plays a minor direct role.

Nonetheless, there is support for the activity of *tat* in the growth of Kaposi sarcoma cells [34]. For example, our group found that recombinant *tat* had stimulatory activity at concentrations of 0.1–1.0 μg/ml. Since we were unable to prove that this activity was not due to endotoxin contamination, we transfected *tat* and mutated *tat* vectors directly into the Kaposi sarcoma cells. While this does not mimic the transcellular activation that is postulated to occur in vivo, it sidesteps the problems of heparin or gelatin binding as well as uptake via RGD receptors. We found that *tat* and not the mutated vector increased growth approximately 1.5- to 2.0-fold in a reproducable manner. In concert with our earlier findings, this increase in proliferation was accompanied with an increase in IL-6-specific mRNA and secreted IL-6. Finally, the effects of *tat* could be abolished with IL-6 antisense constructs, suggesting that part of the mitogenic effects of *tat* could be modulated by IL-6 [34]. While lending credence to a direct role for *tat* in Kaposi sarcoma, additional work in this area by other groups is required before a direct role for *tat* in the etiology of Kaposi sarcoma is accepted.

Other growth factors

Recent studies identify oncostatin-M as a major growth factor for Kaposi sarcoma cells [79]. Oncostatin-M is a T-cell and monocyte-produced cytokine that selectively increases IL-6 expression in endothelial cells [80]. It appears that oncostatin-M can share functional properties of both LIF and IL-6, depending on the cellular receptors present. Oncostatin-M is a potent mitogen for Kaposi sarcoma and induces IL-6 in Kaposi sarcoma cells [17,79]. Oncostatin-M may be a transforming agent, as oncostatin-M alters the histologic characteristics of Kaposi sarcoma cells in culture and supports the growth of these cells in soft agar [79]. In these properties, it is different from other cytokines. Oncostatin-M is the principal T-cell-derived growth factor that was identified earlier [8] in retrovirally infected T cells.

The control and expression of oncostatin-M and its receptor is under intense study. It will be interesting to see if abnormal expression of one of the subtypes of the oncostatin-M receptor can account for the transformation of normal mesenchymal cells. If so, this lesion could explain the differential effects of IL-6, *tat*, and oncostatin-M on Kaposi sarcoma cells compared to normal endothelial or smooth muscle cells.

New directions of research

To date, many potential paths to the development of Kaposi sarcoma have been identified. The pivotal role of IL-6 and its modulation by multiple cytokines, including oncostatin-M, suggests that this cytokine plays a major part in the pathogenesis. Alteration of expression of oncostatin-M receptors

by HIV-*tat* and possibly other sexually transmitted agents is an attractive hypothesis. It also provides for a more important indirect role for *tat*; it could be an amplifier of altered cytokine expression (such as oncostatin-M) from HIV-infected T cells and monocytes.

Indeed, the very nature of the AIDS Kaposi sarcoma progenitor cells has also recently come into question. Work from two groups suggests that adherent cells, with immunologic and phenotypic characteristics similar to mesenchymal Kaposi sarcoma progenitor cells, can be found in the peripheral blood of patients with HIV-associated Kaposi sarcoma [81]. If true, this finding would completely alter our understanding of the nature of the multifocal Kaposi sarcoma tumors. For example, rather than postulating that local production of growth factors such as oncostatin-M, TNF-α, IL-1β, or PDGF would result in the formation Kaposi sarcoma cells, it is postulated that circulating Kaposi sarcoma progenitor cells could implant into tissues and proliferate locally. Infection with HIV and the immune disturbances associated with HIV infection could increase the frequency of these circulating cells. This process could give rise to the spontaneous production of tumors at multiple sites. This provocative hypothesis is currently under study in several laboratories.

New therapeutic agents

The laboratory studies completed in the last several years significantly alter our concept and understanding of the pathogenesis and etiology of Kaposi sarcoma. These studies also provide the basis for new therapeutic interventions that may potentially have activity in modulating the growth of Kaposi sarcoma in vivo. For example, IL-4 is a potent inhibitor of IL-6 expression in monocytes and inhibits IL-6 production and proliferation of Kaposi sarcoma cells [77]. As such, it may have antineoplastic activity in vivo. Other agents, such as recombinant platelet factor-4, also inhibit the proliferation of Kaposi sarcoma cells in vitro [77]. The mitogenic effects of basic fibroblast growth factor (bFGF) on Kaposi sarcoma cells and its inhibition by charged molecules suggest that anionic surfactants, such as pentosan polysulfate or the newly described polysulfated polysaccharide (SPPG) that binds bFGF, could inhibit Kaposi sarcoma cells. Finally, a variety of inhibitors of IL-6 and oncostatin-M have been described [79]. It is possible that inhibitors of these cytokines or receptor-mediated inhibitors of IL-1 or TNF-α could have biologic activity in patients with HIV-associated Kaposi sarcoma. Thus, the in vitro study of the growth of Kaposi sarcoma cells has led to a wide variety of potential therapeutic interventions.

References

1. Mitsuyasu RT: AIDS-related Kaposi's sarcoma: A review of its pathogenesis and treatment. Blood Rev 2:222, 1988.
2. Mitsuyasu RT: Clinical variants and staging of Kaposi's sarcoma. Semin Oncol 14:13, 1987.
3. Reynolds P, Saunders LD, Layefsky ME, Lemp GF: An update on Kaposi's sarcoma reporting in San Francisco (letter). J AIDS 4:825, 1991.
4. Reynolds P, Layefsky ME, Saunders LD, Lemp GF, Payne SF: Kaposi's sarcoma reporting in San Francisco: A comparison of AIDS and cancer surveillance systems. J AIDS 3(Suppl. 1):S8, 1990.
5. Haverkos HW, Friedman-Kien AE, Drotman DP, Morgan WM: The changing incidence of Kaposi's sarcoma among patients with AIDS. J Am Acad Dermatol 22(Suppl.):1250, 1990.
6. Mitsuyasu RT, Groopman JE: Biology and therapy of Kaposi's sarcoma. Semin Oncol 11:53, 1984.
7. Ensoli B, Nakamura S, Salahuddin SZ, Biberfeld P, Larsson L, Beaver B, Wong-Staal F, Gallo RC: AIDS-Kaposi's sarcoma-derived cells express cytokines with autocrine and paracrine growth effects. Science 243:223, 1989.
8. Nakamura S, Salahuddin SZ, Biberfeld P, Ensoli B, Markham PD, Wong-Staal F, Gallo RC: Kaposi's sarcoma cells: Long-term culture with growth factor from retrovirus-infected $CD4^+$ T cells. Science 242:426, 1988.
9. Miles SA, Rezai AR, Salazar-González JF, Meyden MV, Stevens RH, Logan DM, Mitsuyasu RT, Taga T, Hirano T, Kishimoto T, Martínez-Maza O: AIDS Kaposi sarcoma-derived cells produce and respond to interleukin 6. Proc Natl Acad Sci USA 87:4068, 1990.
10. Biberfield P, Nakamura S, Salahuddin ZS, Ensoli B, Gallo RC: Characteristics of in vitro culture of KS-derived cells. Int Conf AIDS 5:600, 1989.
11. Ensoli B, Nakamura S, Salahuddin SZ, Wong-Staal F, Gallo RC: AIDS-Kaposi's sarcoma (KS) derived cells express cytokines with a potential role in the pathogenesis of KS lesions. Int Conf AIDS 5:599, 1989.
12. Friedman-Kien AE, Ostreicher R: Overview of classical and epidemic Kaposi's sarcoma. In: Friedman-Kien AE, Laubenstein LJ (eds): AIDS. The Epidemic of Kaposi's Sarcoma and Opportunistic Infections. New York, Masson, 1984.
13. Friedman-Kien AE, Saltzman BR: Clinical manifestations of classical, endemic African, and epidemic AIDS-associated Kaposi's sarcoma. J Am Acad Dermatol 22(Suppl.):1237, 1990.
14. Ensoli B, Barillari G, Salahuddin SZ, Gallo RC, Wong-Staal F: Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Nature 345:84, 1990.
15. Parravicini C, Kaaya E, Gendelman R, Berti E, Nakamura S, Ensoli B, Gallo RC, Biberfeld P: Fibroblastic and monocytic spindle cells in HIV-related and endemic Kaposi's sarcoma (KS). Int Conf AIDS 7:117, 1991.
16. Ensoli B, Barillari G, Gallo RC: Pathogenesis of AIDS-associated Kaposi's sarcoma. Hematol Oncol Clin North Am 5:281, 1991.
17. Ensoli B, Salahuddin SZ, Gallo RC: AIDS-associated Kaposi's sarcoma: A molecular model for its pathogenesis. Cancer Cells 1:93, 1989.
18. Mitsuyasu RT, Taylor JM, Glaspy J, Fahey JL: Heterogeneity of epidemic Kaposi's sarcoma. Implications for therapy. Cancer 57:1657, 1986.
19. Afrasiabi R, Mitsuyasu RT, Nishanian P, Schwartz K, Fahey JL: Characterization of a distinct subgroup of high-risk persons with Kaposi's sarcoma and good prognosis who present with normal T4 cell number and T4:T8 ratio and negative HTLV-III/LAV serologic test results. Am J Med 81:969, 1986.
20. Friedman-Kien AE: Disseminated Kaposi's sarcoma syndrome in young homosexual men. J Am Acad Dermatol 5:468, 1981.
21. Gottlieb GJ, Ragaz A, Vogel JV, Friedman-Kien A, Rywlin AM, Weiner EA, Ackerman

AB: A preliminary communication on extensively disseminated Kaposi's sarcoma in young homosexual men. Am J Dermatopathol 3:111, 1981.
22. Friedman-Kien AE, Laubenstein LJ, Rubinstein P, Buimovici-Klein E, Marmor M, Stahl R, Spigland I, Kim KS, Zolla-Pazner S: Disseminated Kaposi's sarcoma in homosexual men. Ann Intern Med 96:693, 1982.
23. Stahl RE, Friedman-Kien A, Dubin R, Marmor M, Zolla-Pazner S: Immunologic abnormalities in homosexual men. Relationship to Kaposi's sarcoma. Am J Med 73:171, 1982.
24. Jaffe HW, Choi K, Thomas PA, Haverkos HW, Auerbach DM, Guinan ME, Rogers MF, Spira TJ, Darrow WW, Kramer MA, Friedman SM, Monroe JM, Friedman-Kien AE, Laubenstein LJ, Marmor M, Safai B, Dritz SK, Crispi SJ, Fannin SL, Orkwis JP, Kelter A, Rushing WR, Thacker SB, Curran JW: National case-control study of Kaposi's sarcoma and *Pneumocystis carinii* pneumonia in homosexual men: Part 1. Epidemiologic results. Ann Intern Med 99:145, 1983.
25. Friedman-Kien AE: Epidemic Kaposi's sarcoma: A manifestation of the acquired immune deficiency syndrome. J Dermatol Surg Oncol 9:637, 1983.
26. Marmor M, Friedman-Kien AE, Zolla-Pazner S, Stahl RE, Rubinstein P, Laubenstein L, William DC, Klein RJ, Spigland I: Kaposi's sarcoma in homosexual men. A seroepidemiologic case-control study. Ann Intern Med 100:789, 1984.
27. Friedman-Kien AE: Kaposi's sarcoma: An opportunistic neoplasm. J Invest Dermatol 82:446, 1984.
28. Des Jarlais DC, Stoneburner R, Thomas P, Friedman SR: Declines in proportion of Kaposi's sarcoma among cases of AIDS in multiple risk groups in New York City [letter]. Lancet 2:1024, 1987.
29. Beral V, Peterman TA, Berkelman RL, Jaffe HW: Kaposi's sarcoma among persons with AIDS: A sexually transmitted infection. Lancet 335:123, 1990.
30. Jensen OM, Mouridsen HT, Petersen NS, Jensen KH, Thomsen K, Ulrich K: Kaposi's sarcoma in homosexual men: Is it a new disease [letter]. Lancet 1:1027, 1982.
31. Friedman-Kien AE, Saltzman BR, Cao Y, Nestor MS, Mirabile M, Li JJ, Peterman TA: Kaposi's sarcoma in HIV-negative homosexual men. Lancet 335:168, 1990.
32. Goriano V, Hewlett I, Friedman-Kien A, Tor J, Huang Y, Epstein J: Definitive exclusion of HIV infection in a Kaposi's sarcoma bisexual man. Suggestions on a pathogenic model of KS. Int Conf AIDS 7:78, 1991.
33. Delli Bovi P, Donti E, Knowles DM, Friedman-Kien A, Luciw PA, Dina D, Dalla-Favera R, Basilico C: Presence of chromosomal abnormalities and lack of AIDS retrovirus DNA sequences in AIDS-associated Kaposi's sarcoma. Cancer Res 46:6333, 1986.
34. Miles S, Rezai A, Gaynor R, Magpantay L, Kishimoto T, Martinez-Maza O: HIV-tat increases IL-6 production by and proliferation of AIDS-KS derived cells. Int Conf AIDS 7:55, 1991.
35. Fenoglio CM, McDougall JK: The relationship of cytomegalovirus to Kaposi's sarcoma. In: Friedman-Kien AE, Laubenstein LJ (eds): AIDS. The Epidemic of Kaposi's Sarcoma and Opportunistic Infections. New York, Masson, 1984, 329–336.
36. Huang ES: The role of cytomegalovirus infection in Kaposi's sarcoma. In: Friedman-Kien AE, Laubenstein LJ (eds): AIDS. The Epidemic of Kaposi's Sarcoma and Opportunistic Infections. New York, Masson, 1984, pp 111–26.
37. Andersen CB, Karkov J, Bjerregaard B, Visfeldt J: Cytomegalovirus infection in classic, endemic and epidemic Kaposi's sarcoma analyzed by in situ hybridization. APMIS 99:893, 1991.
38. Hashimoto H, Müller H, Müller F, Schmidts HL, Stutte HJ: In situ hybridization analysis of cytomegalovirus lytic infection in Kaposi's sarcoma associated with AIDS. A study of 14 autopsy cases. Virchows Arch A Pathol Anat Histopathol 411:441, 1987.
39. Drew WL, Huang ES: Etiology of Kaposi's sarcoma: Role of Cytomegalovirus. In: Ziegler JL, Dorfman RF (eds): Kaposi's Sarcoma: Pathophysiology and Clinical Management. New York, Marcel Dekker, 1988, pp 113–28.

40. Siegal B, Levinton-Kriss S, Schiffer A, Sayar J, Engelberg I, Vonsover A, Ramon Y, Rubinstein E: Kaposi's sarcoma in immunosuppression: Possibly the result of a dual viral infection. Cancer 65:492, 1990.
41. Beral V, Bull D, Jaffe H, Evans B, Gill N, Tillett H, Swerdlow AJ: Is risk of Kaposi's sarcoma in AIDS patients in Britain increased if sexual partners came from United States or Africa? [published erratum appears in Br Med J 302(6779):752, 1991]. Br Med J 302:624, 1991.
42. Beral V, Bull D, Jaffe H: Sexual spread of Kaposi's. Nurs Times 87:13, 1991.
43. Lifson AR, Darrow WW, Hessol NA, O'Malley PM, Barnhart L, Jaffe HW, Rutherford GW: Kaposi's sarcoma among homosexual and bisexual men enrolled in the San Francisco City Clinic Cohort Study. J AIDS 3(Suppl. 1):S32, 1990.
44. Lane HC, Davey RT, Jr., Sherwin SA, Masur H, Rook AH, Manischewitz JF, Quinnan GV, Smith PD, Easter ME, Fauci AS: A Phase I trial of recombinant human interferon-gamma in patients with Kaposi's sarcoma and the acquired immunodeficiency syndrome (AIDS). J Clin Immunol 9:351, 1989.
45. Volberding P, Valero R, Rothman J, Gee G: Alpha interferon therapy of Kaposi's sarcoma in AIDS. Ann NY Acad Sci 437:439, 1984.
46. Kovacs JA, Deyton L, Davey R, Falloon J, Zunich K, Lee D, Metcalf JA, Bigley JW, Sawyer LA, Zoon KC, Masur H, Fauci AS, Lane HC: Combined zidovudine and interferon-α therapy in patients with Kaposi sarcoma and the acquired immunodeficiency syndrome (AIDS). Ann Intern Med 111:280, 1989.
47. Rybojad M, Borradori L, Verola O, Zeller J, Puissant A, Morel P: Non-AIDS-associated Kaposi's sarcoma (classical and endemic African types): Treatment with low doses of recombinant interferon-alpha. J Invest Dermatol 95(Suppl.):176S, 1990.
48. Krown SE, Niedzwiecki D, Bhalla RB, Flomenberg N, Bundow D, Chapman D: Relationship and prognostic value of endogenous interferon-alpha, beta 2-microglobulin, and neopterin serum levels in patients with Kaposi sarcoma and AIDS. J AIDS 4:871, 1991.
49. Sawyer LA, Metcalf JA, Zoon KC, Boone EJ, Kovacs JA, Lane HC, Quinnan GV, Jr.: Effects of interferon-alpha in patients with AIDS-associated Kaposi's sarcoma are related to blood interferon levels and dose. Cytokinetics 2:247, 1990.
50. Ensoli B, Barillari G, Salahuddin SZ, Gallo RC, Wong-Staal F: Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Nature 345:84, 1990.
51. De Wit R, Raasveld MHM, Ten Berge RJM, Van der Wouw PA, Bakker PJM, Veenhof CHN: Interleukin-6 concentrations in the serum of patients with AIDS-associated Kaposi's sarcoma during treatment with interferon-alpha. J Intern Med 229:539, 1991.
52. Miles SA, Rezai AR, Logan D, Salazar-Gonzalez JF, Vander Meyden M, Mitsuyasu RT, Taga T, Hirano T, Kishimoto T, Martinez-Maza O: AIDS Kaposi's sarcoma-derived cells produce and respond to interleukin 6. Int Conf. AIDS. 6:112, 1990.
53. Weich HA, Salahuddin SZ, Gill P, Nakamura S, Gallo RC, Folkmann J: AIDS-Associated Kaposi's sarcoma-derived cells in long-term culture express and synthesize smooth muscle alpha-actin. Am J Pathol 139:1251, 1991.
54. Werner S, Hofschneider PH, Stürzl M, Dick I, Roth WK: Cytochemical and molecular properties of simian virus 40 transformed Kaposi's sarcoma-derived cells: Evidence for the secretion of a member of the fibroblast growth factor family. J Cell Physiol 141:490, 1989.
55. Sinkovics JG: Kaposi's sarcoma: Its 'oncogenes' and growth factors. Crit Rev Oncol Hematol 11:87, 1991.
56. Wellstein A, Zugmaier G, Califano JA, III, Kern F, Paik S, Lippman ME: Tumor growth dependent on Kaposi's sarcoma-derived fibroblast growth factor inhibited by pentosan polysulfate. J Natl Cancer Inst 83:716, 1991.
57. Buonaguro FM, Galloway DA, Beth-Giraldo E, Giraldo G, McDougall J: Kaposi's sarcoma associated transforming sequences (meeting abstract). Presented at the Second International Symposium on AIDS and Associated Cancers in Africa, October 7–9, 1987.

58. Buonaguro FM, Giraldo G, Nakamura Y, Vecchione R, Alessi E, Finzi AF, Safai B, Mueller N, Hatzakis A, Owili DM: Genetic mechanisms in Kaposi's sarcoma pathogenesis. Int Conf AIDS 7:117, 1991.
59. Spandidos DA, Kaloterakis A, Yiagnisis M, Varatsos A, Field JK: Ras, C-myc and C-erbB-2 oncoprotein expression in non-AIDS Mediterranean Kaposi's sarcoma. Anticancer Res. 10:1619, 1990.
60. Lo SC, Liotta LA: Vascular tumors produced by NIH/3T3 cells transfected with human AIDS Kaposi's sarcoma DNA. Am J Pathol 118:7, 1985.
61. Salahuddin SZ, Nakamura S, Biberfeld P, Kaplan MH, Markham PD, Larsson L, Gallo RC: Angiogenic properties of Kaposi's sarcoma-derived cells after long-term culture in vitro. Science 242:430, 1988.
62. Albini A, Nakamura S, Poggi L, Gallo RC, Salahuddin SZ, Thompson EW: Cultured AIDS-related Kaposi's sarcoma cells (AIDS-KS) produce activators of endothelial cell chemotaxis and invasiveness. Int Conf AIDS 7:118, 1991.
63. Werner S, Hofschneider PH, Roth WK: Cells derived from sporadic and AIDS-related Kaposi's sarcoma reveal identical cytochemical and molecular properties in vitro. Int J Cancer 43:1137, 1989.
64. Werner S, Viehweger P, Hofschneider PH, Roth WK: Low mitogenic response to EGF and TGF-alpha: A characteristic feature of cultured Kaposi's sarcoma derived cells. Oncogene 6:59, 1991.
65. Werner S, Hofschneider PH, Heldin C-H, Östman A, Roth WK: Cultured Kaposi's sarcoma-derived cells express functional PDGF A-type and B-type receptors. Exp Cell Res 187:98, 1990.
66. Roth WK, Werner S, Schirren CG, Hofschneider PH: Depletion of PDGF from serum inhibits growth of AIDS-related and sporadic Kaposi's sarcoma cells in culture. Oncogene 4:483, 1989.
67. Podor TJ, Jirik FR, Loskutoff DJ, Carson DA, Lotz M: Human endothelial cells produce IL-6. Lack of responses to exogenous IL-6. Ann NY Acad Sci 557:374–85, 1989.
68. Ammann AJ, Palladino MA, Volberding P, Abrams D, Martin NL, Conant M: Tumor necrosis factors alpha and beta in acquired immunodeficiency syndrome (AIDS) and aids-related complex. J Clin Immunol 7:481, 1987.
69. Ensoli B, Buonaguro L, Barillari G, Gallo RC: Biological properties of tat, the transactivator gene of HIV-1. Int Conf AIDS 7:55, 1991.
70. Vogel J, Hinrichs SH, Reynolds RK, Luciw PA, Jay G: The HIV tat gene induces dermal lesions resembling Kaposi's sarcoma in transgenic mice. Nature 335:606, 1988.
71. Hsu M-C, Schutt AD, Holly M, Slice LW, Sherman MI, Richman DD, Potash MJ, Volsky DJ: Inhibition of HIV replication in acute and chronic infections in vitro by a Tat antagonist. Science 254:1799, 1991.
72. Marciniak RA, Calnan BJ, Frankel AD, Sharp PA: HIV-1 Tat protein *trans*-activates transcription in vitro. Cell 63:791, 1990.
73. Mann DA, Frankel AD: Endocytosis and targeting of exogenous HIV-1 Tat protein. EMBO J 10:1733, 1991.
74. Ensoli B, Barillari G, Salahuddin SZ, Gallo RC, Wong-Staal F: HIV-1 Tat protein released during acute infection stimulates growth of spindle cells derived from AIDS-Kaposi's sarcoma. Int Conf AIDS 6:202, 1990.
75. Frankel AD, Biancalana S, Hudson D: Activity of synthetic peptides from the Tat protein of human immunodeficiency virus type 1. Proc Natl Acad Sci USA 86:7397, 1989.
76. Calnan BJ, Biancalana S, Hudson D, Frankel AD: Analysis of arginine-rich peptides from the HIV Tat protein reveals unusual features of RNA-protein recognition. Genes Dev 5:201, 1991.
77. Miles SA, Rezai A, Kishimoto T, Mitsuyasu RT, Martínez-Maza O: Multiple cytokines simultaneously alter secretion of interleukin-6 and growth of AIDS Kaposi's sarcoma cells. Submitted, 1992.
78. Lunardi-Iskandar Y, Lam HH, Judde JG, Gallo RC: Cytokine and hormone receptors on

AIDS-Kaposi's sarcoma (KS)-derived cells. Meeting of the Laboratory of Tumor Cell Biology, Bethesda, MD. (abs. Tues. 7), 1991.
79. Miles SA, Martiñez-Maza O, Rezai A, Magpantay L, Salahuddin S, Nakamura S, Radka S, Linsley P: Oncostatin-M as a potent mitogen for AIDS-Kaposi sarcoma derived cells. Submitted, 1992.
80. Brown TJ, Rowe J, Jingwen L, Shoyab M: Regulation of IL-6 expression by Oncostatin-M. J Immunol 147:2175–2180, 1991.
81. Looney DJ, Poeschla E, Fiegal E, Badel P, Rappaport J, Wong-Staal F: Spindle like cells derived from the peripheral blood of patients with Kaposi's sarcoma. Meeting of the Laboratory of Tumor Cell Biology, Bethesda, MD (abs. Tues. 9), 1991.
82. Berman MA, Sandborg CI, Calabia BS, Andrews BS, Friou GJ: Interleukin 1 inhibitor masks high interleukin 1 production in acquired immunodeficiency syndrome (AIDS). Clin Immunol Immunopath 42:133–40, 1987.

7. BCL-2: Physiology and role in neoplasia

Adam Bagg and Jeffrey Cossman

Introduction

Deregulation of proto-oncogenes or inhibition of tumor suppressor genes results in the acquisition of a cellular growth advantage, usually manifested as increased proliferation [1]. Many of the mechanisms that mediate these functions have been elucidated, while the function of others still remains to be resolved. Nevertheless, there exists a broad stratification of genes whose alteration is mechanistic in the development of neoplasia. By contrast, *bcl*-2, which was originally identified in 1984 [2], appears to function by a mechanism independent of the above two categories, and it may be the first described member of a third broad class of genes whose deregulation plays a role in oncogenesis. It appears to act by inhibiting cell death, rather than by stimulating proliferation.

The demonstration of proto-oncogenes first derived from observations of the transforming capacity of retrovirally transmitted oncogenes or in vitro transformation assays. Alternative clues to the identification of putative proto-oncogenes are the nonrandom cytogenetic abnormalities, as these have revealed the location of genes involved in the pathogenesis of neoplasia. The most common translocations observed in lymphoid malignancies, which encompass the non-Hodgkin's lymphomas (NHL), involve the loci of the antigen receptor (both immunoglobulin and T-cell receptor) genes; the cloning of the reciprocal breakpoints has uncovered numerous 'novel,' and previously unrecognized, genes [3]. This, too, was the case in follicular NHL, in which a t(14;18) cytogenetic translocation has been identified morphologically in the majority of cases. Using the immunoglobulin heavy chain gene (on 14q32) as a handle, the cloning of the chromosome 18 breakpoint led to the identification of the *bcl*-2 locus (for *B*-cell *l*ymphoma/leukemia). The numeric designation relates to the fact that the locus involved in the t(11;14) translocation, reported on just prior to the above description, had been designated *bcl*-1 [4].

While most of the initial data on *bcl*-2 derived from studies on its involvement in the t(14;18) translocation, a significant amount of information has subsequently emerged on the normal or physiological role of the gene. This

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review will, therefore, discuss the data in an historically inverse fashion, but in a way that attempts to evolve logically, from physiology through to pathology.

BCL-2 physiology

Gene structure and expression

Structure. The normal *bcl*-2 gene is located on the long arm of chromosome 18, at band q21.3, in close (genomic) proximity to the plasminogen activator inhibitor type 2 gene [5]. It comprises three exons, the first of which is noncoding, due to the presence of stop codons in all three open reading frames [6]. Indeed, the gene has long untranslated regions at both the 5′ and 3′ ends, with the open reading frame spanning the 3′ end of exon 2 and the 5′ end of exon 3. Pulse-field gel electrophoresis studies initially suggested that the gene was several hundred kilobases long [7]. Only recently has the entire genomic clone of *bcl*-2 been isolated, using meiotic recombination between yeast artificial chromosomes (YACs), which demonstrated that the gene spans approximately 230 kb [8]. The first intron is only 220 bp long, while the intron between exons 2 and 3 was thought to be one of the largest known (~370 kb), based on pulse-field gel electrophoresis analysis [7]. However, it has been demonstrated that the second intron is 'only' 225 kb long [8].

Regulation. There exist two alternative promoters, with differential (two vs. three) exon usage, but our knowledge of the factors involved in the normal regulation of transcription is limited. The regulation of the *bcl*-2 gene appears to be fairly complex, with transcription being affected by both stimulatory as well as inhibitory factors [9]. One promoter is located immediately 5′ to the ORF in exon 2 (classic TATA plus CAAT box, as well as a decanucleotide, ATGCAAAGCA, which is homologous with the tissue-specific immunoglobulin-variable region enhancers, referred to as the 'dc/cd sequence', and an SV40 enhancer), while the other is further 5′ in exon 1 (this region is GC rich and contains seven Sp1-binding motifs, as noted in SV40, but no TATA box). The latter promoter region is similar to that noted in other oncogenes, such as *abl* and *myb*. Experiments using cyclohexamide provided indirect evidence that short-lived transacting factor(s) may be involved in the regulation of transcription [10]. Other factor(s) may act to destabilize or degrade the *bcl*-2 message. The methylation status of the 5′ end of the gene does not appear to affect transcription. In a proliferating T-cell leukemic line, unstimulated cells express high levels of *bcl*-2 mRNA, but these diminished rapidly following the cessation of growth induced by phorbol esters [11]. As a consequence of differential exon utilization (due to the presence of alternate promoters), alternate

splicing, and different polyadenylation sites, more than one transcript results. The mRNA species that are generated are 8.5, 5.5, and 3.5 kb in length. The transcripts have a relatively short half-life of 2.5 hours, perhaps related to the presence of AU-rich sequences in the 3′ UT region. Transcript levels are highest during the pre-B-cell stage of development, with down-regulation during maturation [7,9].

***bcl*-2 protein.** Until recently, the subcellular localization and function of the *bcl*-2 protein had been enigmas. The absence of signal peptides from sequence data [12,13] implied that the protein was intrinsic to the cell and not exported. Initial DNA sequence analysis failed to show any significant homology with any known oncogenes, but it did appear to have some similarity to an Epstein-Barr virus protein, BHRF1 [12,14]. This provided the first hint of what may evolve into a tantalizing link between *bcl*-2 and EBV (see later). BHRF1, a component of the early antigen (EA) complex of EBV, is a protein of unknown function, which is expressed in the initial stages of viral infection, and it does not appear to be involved in the development of EBV latency. Two proteins are generated from the *bcl*-2 mRNA species, a ~25- to 26-kDa (239 amino acid) α *bcl*-2 and a 22-kDa (205 amino acid) β *bcl*-2, with the former predominating. These two proteins have identical 196 amino terminus amino acids [13], with the carboxy terminus divergence being a consequence of the alternative splice site selection.

The observation of a hydrophobic/lipophilic carboxy-terminus domain suggested that the protein may be subcellularly localized to membranes [15], with the carboxy-terminus tail anchoring *bcl*-2 into membranes. Indeed, biochemical studies revealed that *bcl*-2 was an integral membrane protein, being ubiquitous in all membranes of the cell, although it appeared to be more abundant in the perinuclear endoplasmic reticulum and nuclear envelope [16]. These initial topographic observations, with its subcellular localization on the cytoplasmic surface of cellular membranes, were reminiscent of that for a number of other oncogenic proteins, including *ras*, *abl*, and other kinases. It was thus thought that the protein perhaps had a role in signal transduction, possibly transmitting signals from growth factor receptors. This notion was consistent with the description of *bcl*-2 being an early response gene, following mitogenic stimulation of lymphocytes [9]. Recently, studies with a hamster monoclonal antibody and cell fractionation experiments have shown that the *bcl*-2 protein is situated in the inner mitochondrial membrane [17]. This fairly conclusive evidence of its subcellular localization makes it novel among protooncogenes.

Cellular expression. Although *bcl*-2 was initially identified and studied most exhaustively in follicular NHL and was thought to be specific for cells with the t(14;18) translocation [18] (see below), it has become clear that the expression of the protein is neither specific for neoplastic cells that carry the

t(14;18) translocation, nor is its expression limited to B cells [19,20]. The description of and immunohistochemical examination with a variety of monospecific and polyspecific antibodies [17,19], has revealed that the protein is expressed in a variety of hematopoietic and nonhematopoietic cells. This expression appears to correlate with the level of maturation, as well as the function and longevity of the cells. Within lymph nodes, *bcl*-2 expression is most abundant in B cells of the follicular mantle, while it is essentially absent from cells within the follicle itself, other than those in the apical light zone [21]. The mantle zone is composed primarily of long-lived, recirculating IgM/IgD$^+$ B cells, while the apical light zone is the area where B cells with high affinity to presented antigen are selected. Interfollicular cells express intermediate levels of the protein. In the thymus, more mature medullary thymocytes demonstrated substantial amounts of *bcl*-2, while less differentiated cortical thymocytes are negative. It is within the cortex that thymocytes undergo positive and negative selection, based upon their T-cell receptor specificities.

The protein is also expressed in nonlymphoid hematopoietic precursors, being found most abundantly in early identifiable myeloid cells, including blasts, promyelocytes, and myelocytes. As these cells mature through the metamyelocyte and band stages, *bcl*-2 protein levels decrease and mature neutrophils have virtually undetectable amounts [20,21]. Not surprisingly, *bcl*-2 expression is not restricted to hematopoietic and lymphoid cells. It has been found in some, but not all, tissues that are either long lived, pluripotent, or hormone/growth factor dependent [21]. Such cells include those in breast, thyroid, pancreatic, and prostatic epithelia, as well as in skin and gastrointestinal epithelia, localized to cells that are long lived and probably self-renewing. This hierarchical expression of the protein correlates very neatly with what is now known about its function (see below). Neurons, which are long-lived postmitotic cells, also express *bcl*-2. However, not all long-lived cells are positive for *bcl*-2; muscle and other tissues of mesodermal origin were found to be negative [21].

Function

Initial clues as to the function of *bcl*-2 derived from two broad approaches, namely, that of transfection experiments and the creation of transgenic mice. The aforementioned immunohistochemical studies, with the anatomic restriction of *bcl*-2 to cells that are long lived and its absence from cells that are dying corroborated the in vitro and transgenic findings.

Transfected *bcl*-2. Under the effect of different promoters, the cloned *bcl*-2 gene has been introduced into a variety of cell lines, including B-lymphoid, myeloid, and fibroblastic lines. In EBV-transformed human lymphoblastoid lines, the effect was an at least threefold increased in clonogenicity in soft agar [22], but this was insufficient to confer tumorigenesis when injected

into immunodeficient mice [23]. By contrast, similar introduction of the gene into murine precursor B cells did not affect proliferation [24]. This apparent disparity may be due to two differences in these two systems, namely, the species of the transfected cells and their degree of maturation. Furthermore, EBV-transformed cells may not be an appropriate system in which to study *bcl*-2, given the fact that EBV itself may induce *bcl*-2 expression [25] (see below).

To answer the question as to whether *bcl*-2 is involved in some of the growth factor pathways, a murine *bcl*-2 gene was introduced into a variety of factor-dependent hematopoietic cell lines [26]. No long-term growth factor independence resulted in IL-2, IL-3, and IL-6 dependent cell lines. However, the consistent effect of the gene appeared to be to extend the survival and spare some cells from death, in particular, IL-3 dependent pro-B lymphocytes, promyelocytes, and mast cells. A similar effect was noted in cells maintained in GM-CSF or IL-4. The effect was not noted, however, in IL-2 dependent T cells or IL-6 dependent myeloma cells. These data suggested that *bcl*-2 interfered with cell death but in a cell-type or factor-restricted fashion. The improved survival of cells transfected with *bcl*-2 is not restricted to growth factor deprivation, but is also observed in response to a variety of other stresses, such as heat shock and exposure to methotrexate [27]. Transfecting NIH3T3 cells led to the increased coupling of growth factor receptors to stimulate inositol phosphate production, strongly supporting a role for *bcl*-2 in the growth-factor-receptor-mediated signal transduction pathway [28]. A report that *bcl*-2 is a GTP-binding protein seemed to confirm its role in signal transduction [29]. This finding, however, was not reproduced in another laboratory, which showed that *bcl*-2 was incapable of binding GTP, that several small GTP-binding proteins were found to be ubiquitous and not to vary with levels of *bcl*-2 expression, and that *bcl*-2 could be separated from these proteins by immunological, electrophoretic, and cell fractionation techniques [30].

Additional gene transfer experiments have illustrated that *bcl*-2 alters the growth characteristics of human B lymphocytes by enhancing their growth in the presence of reduced serum and in limiting dilution cultures [31], as well as showing an effect of enhancing tumorigenicity of NIH3T3 cells [32], which was also shown to be complemented by cotransfection with Ha-*ras* in another rodent fibroblast system [33]. Other cotransfection experiments have repeatedly demonstrated synergy between *bcl*-2 and *myc*, in both B cells [22,24,31] and T cells [34]. These in vitro observations have been confirmed in both transgenic studies and in the clinical setting (see below).

***bcl*-2 Transgene.** The in vitro observations on the effect of *bcl*-2 were extended when its function was evaluated in an intact immune system through the generation of transgenic mice. Animals bearing the *bcl*-2-Ig minigene, representing that seen in follicular NHL, revealed overexpression of the gene in splenic and thymic tissue [35,36]. Cellular and immunologic

characterization showed that there was an expanded population of small resting and recirculating IgM/IgD$^+$ B cells, which resided in G_0/G_1. Although resting, the cells were functionally normal in terms of their response to LPS and anti-μ, and they also had an enhanced secondary immune response. This latter observation indicates that *bcl*-2 may have a significant physiologic role in the emergence and maintenance of B-cell memory [37]. Initially, the B-cell expansion was indolent and polyclonal, but over a period of time oligoclonality manifested, with the eventual progression to malignant lymphomas. Half of these disseminated, monoclonal, immunoblastic lymphomas had rearranged *myc* genes [38]. These phenomena suggested that prolonged B-cell life increased tumor incidence.

Transgenic mice generated with the *bcl*-2 gene under the control of the 5′ IgH enhancer (Eμ-*bcl*-2 transgene) exhibited abnormalities of both B cells and T cells [39]. While the observations with the *bcl*-2-Ig minigene were initially similar (with an increase in the number of B cells with an enhanced survival capacity), these mice did not develop B-cell lymphomas. Indeed, many instead developed a systemic autoimmune-like disease, resembling systemic lupus erythematosis [40]. The reason for this difference may relate to the differences in the genetic backgrounds of the mice and/or the nature of the constructs. The T-cell effects were the exhibition of prolonged survival ex vivo. The capacity for *bcl*-2 to synergize with other oncogenes was investigated in doubly transgenic mice [41]. Mice bearing both *myc* and *bcl*-2 developed tumors much more rapidly than those carrying *myc* alone. Interestingly, these tumors had a novel immature phenotype, which may represent a neoplastic counterpart of a lymphoid committed stem cell.

Apoptosis. A detailed study of the mechanism of death in the IL-3 deprived cells, noted in the transfection experiments above, clearly showed that the cells were dying by apoptosis [17]. These dying cells demonstrated plasma membrane blebbing, volume loss, nuclear condensation, and endonucleolytic cleavage of DNA into 180-bp oligonucleosomal fragments. The presence of the transfected *bcl*-2 blocked this programmed cell death. Therefore, it appears from the transfection and transgenic studies that *bcl*-2 functions to inhibit apoptosis and that on its own it is not tumorigenic, but that by enabling B cells to live longer it puts them at risk for other genetic events, with *myc* not uncommonly being the target, which then leads to the development of neoplasia. Furthermore, the observation of *bcl*-2 expression in nonhematopoietic tissues, noted in the previous section, supports this notion, in that cancers of the skin, colon, breast, prostate, and pancreas rank first, third, fourth, fifth, and ninth, respectively, in the incidence of all carcinomas [21].

Apoptosis is a physiologic control mechanism in a number of processes, including embryonic development and clonal deletion in the immune system [42,43]. A variety of physiological mechanisms exist in at least some cell types to prevent this programmed cell death, with *bcl*-2 being an important,

but probably not the only, component. The effect of transfecting individual EBV latent genes on the induction of apoptosis has been examined. Intriguingly, expression of the EBV latent membrane protein, LMP1, suppressed apoptosis, and this correlated with the endogenous expression of *bcl*-2 [25]. It appears that elevated *bcl*-2 expression may normally rescue antigen-reactive B cells as part of the selection mechanism implicated in the development of antibody affinity maturation [44]. In follicular NHL and transgenic mice, the inappropriately high levels of *bcl*-2 expression allow B cells to bypass this physiologic control mechanism and thus to accumulate (by inhibiting cell loss). In both of these scenarios, additional factors, in particular *myc* activation, are required for the development of more aggressive hematopoietic malignancies. Centrocytes, the putative normal equivalent of the neoplastic cells in follicular NHL, undergo apoptotic cell death unless they are rescued by the stimulation of their CD40 receptors and surface immunoglobulin [45]. In follicular NHL, reduced apoptotic cell death has been observed [46], which is almost certainly due to increased levels of *bcl*-2 (see next section). CD40 is a member of a recently described superfamily of cell surface proteins related to the NGF receptor [47]. Of interest, an anti-*fas* antibody, which resembles the Apo-1 antibody (which is a monoclonal antibody that induces apoptosis in lymphoid cell lines [48]), also induces apoptosis in hematopoietic cells and fibroblasts [49]; *fas*, a transmembrane protein, has significant homology with the TNF receptor, which is also a member of the NGF receptor family. Despite its name, when TNF (tumor necrosis factor) binds to its specific receptor, it can induce apoptosis [50], and TNF receptors have been observed on some lymphoma cells [51]. p53, a prototypic tumor suppressor gene, can function to induce apoptosis [52], and it may be involved in the pathogenesis of some lymphomas [53]. A variety of other, and as yet undefined, mRNAs are associated with the induction of apoptosis [54], and recently CD77, a neutral glycolipid, has been identified as an antigen of germinal center B cells entering apoptosis [55]. Clearly then, there are complex pathways, yet to be unravelled, involved in the process of programmed cell death. Nevertheless, *bcl*-2 appears to be a major component in the inhibition of this pathway. The subcellular localization of the *bcl*-2 protein in the inner mitochondrial membrane suggests that inhibition of apoptosis may involve oxidative phosphorylation and/or electron and metabolite transportation [17].

bcl-2 in disease

Follicular NHL

Although the *bcl*-2 gene was initially cloned from an acute B-cell leukemic line [2], the prototypic disease in which it has been extensively studied is follicular NHL. The t(14;18)(q32;q21) is the most common translocation

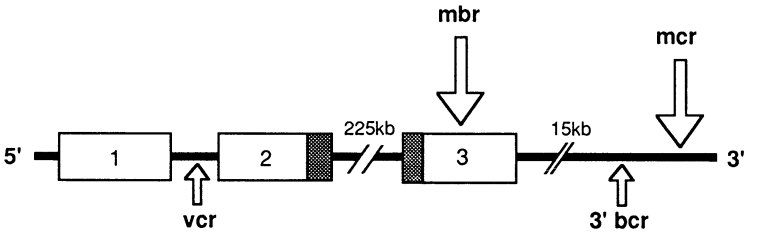

Figure 1. bcl-2 breakpoint cluster regions. The alternatively used first two exons of the *bcl-2* gene are followed by a long second intron of approximately 225 kb. The open reading frame of the gene (shaded) is contained in the 3′ end of the second exon and the 5′ end of the third exon. The four clustered breakpoint regions are designated as vcr (variant or 5′ cluster region), mbr (major breakpoint region), 3′ bcr (3′ breakpoint cluster region) and mcr (minor cluster region). The frequency of involvement of these breakpoints has been found to be: vcr (5–10%), mbr (60%), 3′ bcr (5–10%) and mcr (20–30%). Note that none of the breakpoints affects the coding region of the gene.

seen in human lymphoid malignancies, and approximately 85% of follicular NHLs carry this translocation [56]. Some studies have, however, questioned the notion that the t(14;18) translocation is 'the hallmark' of follicular NHL [57]. The incidence of the translocation appears to vary with geographic locale and ethnicity, being found in only 41% of European follicular NHLs [58], 27% of Japanese patients with the disease [59], and 25–57% of Hong Kong Chinese follicular NHLs [60,61]. Despite this variation in reported incidence, there remains a clear association between the translocation and follicular NHL.

The breakpoints on the chromosome 18 cluster (Fig. 1). The most common site involved is in the 3′ UT region of exon 3 of *bcl-2*, in which the breakpoints are clustered in a region approximately 150 bp long. This site is referred to as the major breakpoint region (*mbr*) [62–64] and accounts for the site of translocation in about 60% of cases. The second commonest area of breakage is in a 500-bp region 20–30 kb 3′ of exon 3, known as the minor cluster region (*mcr*), and is involved in 25% of breakpoints [65–67]. Rarely, the rearrangements involve regions 5′ of the *bcl-2* gene [68]. Alterations in the 5′ area have also been reported to coexist with *mbr* and *mcr* rearrangements [69]. Most often, the reciprocal translocation involves a breakage at the Ig heavy chain locus, within the 5′ portion of one of the J_H segments. In rare instances, the D_H region is juxtaposed with the *bcl-2* gene [70,71]. Also, translocations involving the loci of the Ig light chain genes, with t(2;18) and t(18;22) translocations have been reported [72,73]. The translocations between the *bcl-2* oncogene and all three Ig gene loci are reminiscent of that which occurs with the *myc* oncogene in Burkitt's lymphoma. Curiously, there is a striking correlation with the chromosome 18 breakpoint in translocations involving the Ig light chain genes, occurring in a short (2.5 kb) region 5′ of the *bcl-2* gene. Based on this, it has recently been proposed that

the region is also an area of clustered breakpoints, being referred to as the variant cluster region (*vcr*) [74]. A t(18;22) translocation has also been reported as a secondary phenomenon in a lymphoma [75], at variance with the typical t(14;18), which has not been reported to occur as a secondary change. In Japanese patients, it has been suggested that the translocations occur at a later stage of B-cell development, as evidenced by the observation of some cases involving either the Ig light chain loci or the D_H Ig heavy chain locus [73,76].

The involvement of the Ig J regions in the translocation provided a hint that the translocation occurs in a precursor stage of B-cell development, at the time of physiological VDJ rearrangement. The observation of extra nucleotides inserted at the joining site [77], reminiscent of 'N' regions, inserted by the nuclear enzyme terminal deoxynucleotidyl transferase (Tdt), supported the hypothesis that translocation occurs due to a mistake in VDJ joining [78]. This has led to the notion that a low but constant background of tumors affecting the lymphoid system may be perceived as an 'acceptable cost of the capacity of the recombinase system to generate immune diversity' [79]. Since the translocation is thought to occur during IgH gene rearrangement, this event almost certainly occurs in bone marrow cells, but only subsequently manifests in peripheral lymphoid tissues once surface Ig is expressed on the cells [80]. Thus, while these rearrangements probably occur early in B-cell ontogeny, it has recently been shown that the recombinase system may also be active in later stages of lymphoid development [81], and also, gene rearrangements can occur in mature B cells, and perhaps also then in follicular NHL cells [82].

However, despite this compelling evidence that the VDJ recombinase mediates the translocation, and the observation of 'signal-like' sequences in close proximity to the chromosome 18 breakpoint [83], the exact mechanism remains controversial, as only imperfect heptamer-nonamer signal sequences have been detected around the *bcl*-2 breakpoints. The breakage on chromosome 18 may be a consequence of DNA repair following single-strand breaks [77]. Another explanation proposed includes the finding of *chi* sequences (sequences that are similar to a prokaryotic activator of recombination) around the *bcl*-2 *mbr*. It appears that slight and inheritable variations in this region may influence the probability of a translocation [84,85]. Analysis of the DNA around the 5′ breakpoints revealed the presence of multiple alternating purine-pyrimidine elements (potential Z-DNA), which may be involved in mediating translocations affecting this region [86].

Whichever translocation occurs, the coding region of the *bcl*-2 gene is left intact, so that the *bcl*-2/Ig fusion transcript encodes a normal *bcl*-2 protein [6,87]. The consequence of the translocation is logfold increases in *bcl*-2 mRNA and protein, indicative of a marked deregulation of the gene following the translocation [6,16,66,87]. While a fusion transcript has been repeatedly detected in cell lines with *mbr* rearrangements (the only *bcl*-2

rearrangement occurring within an exon), only recently was this chimeric mRNA detected in primary human follicular NHL [88]. It is not entirely clear what mediates the increased *bcl*-2 expression that is observed following the translocation. It has been suggested that the 'proximity' of the IgH enhancer may result in increased transcription, but the size of the second intron in the *bcl*-2 gene implies that it would have to act over a considerable distance. Loss of AU-rich sequences from the 3′ end of the *bcl*-2 gene, as a consequence of the 3′ translocation, may enhance mRNA stability [89–91]. Sequences other than AU-rich regions may also affect message stability [92]. The half-life of the transcript encoded by the translocated *bcl*-2, however, does not appear to be that different from the wild-type gene [6,7], arguing against this as an important consequence of the translocation. However, the correct determination of a half-life for mRNA may not be feasible using only one method and may require a variety of approaches [93]. Regulation may also be altered at the translational level, as a result of the translocation, since UT regions both 5′ [94] and 3′ [95] of other messages, such as those for TGF-β and creatine kinase, respectively, demonstrate the ability to bind factors that regulate translation. While the precise mechanism remains elusive, it appears that quantitative changes in *bcl*-2 are more important than qualitative alterations, in that no amino acid altering mutation of the gene have been found in primary human follicular NHL [96,97], despite the observation of point mutations in two potentially critical regions in cell lines [6,87].

Involvement of bcl-*2 in other malignancies*

Increased expression of *bcl*-2, with or without translocations involving the *bcl*-2 locus, is not restricted to follicular NHL. Approximately 20–30% of diffuse B-cell non-Hodgkin's lymphomas have translocations involving *bcl*-2, as detected by conventional cytogenetics, Southern blotting, or the polymerase chain reaction (PCR) [64,67,98,99]. Although it was once proposed that the expression of the *bcl*-2 protein was a specific marker for B-cell malignancies harboring the t(14;18) translocation [18], it has subsequently been shown that the expression of the protein is independent of, and certainly not specific for, the translocation [19,20]. Thus, although *bcl*-2 expression increases in tumors with the t(14;18), its expression can also be detected in the absence of the translocation, in other lymphoproliferative disorders, plasma cell dyscrasias, chronic myelogenous leukemia, as well as in normal T and B lymphocytes and nonhematopoietic tissues [19–21,100].

In B-cell chronic lymphocytic leukemia (CLL), the *bcl*-2 gene is rearranged in fewer than 10% of patients [101–104]. In almost all the cases analyzed, the breakpoints on chromosome 18 cluster in the 5′ region of the gene, and all the rearrangements result in the juxtaposition with the Ig light chain genes. These translocations are very similar, if not identical with, those in follicular NHL involving the *vcr* [74]. Whereas the typical *bcl*-2 rearrange-

ments affecting the 3′ region of the gene result in a head-to-tail juxtaposition with the Ig gene, the translocations seen in CLL result in a head-to-head configuration. No 'N' nucleotides are present in these translocations, consistent with the fact that Tdt is not active at the time of Ig light chain gene rearrangement. Z-DNA may be involved in mediating the translocation in this setting [86]. Whatever the mechanism, the association of *bcl*-2 rearrangements involving Ig light chain genes suggests that the translocation occurs at a later stage of B-cell ontogeny as compared with most follicular NHL. An additional variant translocation has been described in small cell lymphocytic NHL, a disease similar to CLL, namely, a novel t(11;18) translocation [105].

In Hodgkin's disease, the pathogenesis, cell of origin, and clonality remain unresolved, related primarily to the paucity of the putative neoplastic cell, the Reed-Sternberg cell, in the tissues of patients with this disease. Using PCR (see section on diagnosis), a number of groups have searched for evidence of the t(14;18) in this disease. In our initial report, 32% of unselected patients with Hodgkin's disease had molecular evidence of the translocation, while this was not found in T-cell NHL, benign lymph nodes, and normal peripheral blood lymphocytes [106]. Although the t(14;18) translocation had previously been identified karyotypically in Hodgkin's disease [107], the high incidence of *bcl*-2-J_H joining detected by PCR was unexpected. Accordingly, the possibility of false-positive PCR results was carefully evaluated. The results were reproduced three times, and with each analysis negative controls, normal lymphocytes, reactive lymph nodes, and T-cell lymphomas remained negative. Thus, the finding of a 32% prevalence of *bcl*-2 rearrangement in Hodgkin's disease appeared not to be an artifact of PCR. In this study, only the *mbr* was analyzed, leaving open the possibility that *bcl*-2 rearrangements might have occurred at other sites in cases thought to be negative. It is not known which cells in the Hodgkin's tissues contain the t(14;18), and an initial survey suggested that the Reed-Sternberg cells do not overexpress *bcl*-2 protein [19,20], but we have detected the protein in the Reed-Sternberg cells of 7 of 7 cases of Hodgkin's disease, using frozen-section immunohistochemistry and a monoclonal antibody. The differences in these results may be a consequence of tissue fixation, antibody titer, or other technical factors. Whether the *bcl*-2 gene is rearranged *and* expressed in Reed-Sternberg cells remains an intriguing question deserving further research. While some other laboratories have confirmed this observation [108–110], others have not [111,112]. The failure to detect rearrangements may be due to technical factors, including the use of paraffin-imbedded tissue, biotinylated, rather than ^{32}P-labelled probes, mismatched or short primers, as well as the selection of certain histologic subtypes. These conflicting results may be a consequence of differences in sensitivity of detection of a rare event, or that Hodgkin's disease may be an even more heterogeneous disease than was originally believed.

A significant body of evidence, based on epidemiologic, serologic,

immunologic, Southern blotting, PCR, and in situ studies, has emerged linking EBV infection with Hodgkin's disease [113–115]. The aforementioned studies, demonstrating PCR-detectable t(14;18), suggest that the activation of *bcl*-2 may play a role in the genesis of the disease, and this may be associated with the recent demonstration that the expression of an EBV gene, LMP1, can induce *bcl*-2, accompanied by the inhibition of apoptosis [25]. These findings raise the possibility that Hodgkin's disease is a disease in which an association between EBV and *bcl*-2 exists. The LMP gene has transforming potential and is found to be expressed in more than a third of patients with Hodgkin's disease [116,117], with the expression being detected exclusively in Reed-Sternberg and Hodgkin's cells, which are the probable neoplastic cells in this disease.

The *bcl*-2 gene has also been examined in a variety of other lymphoproliferative disorders. Rearrangements are found, as expected, in extranodal follicular NHLs [109], but they are extremely rare in other extranodal NHLs, in particular, in so-called MALTomas (lymphomas of Mucosa Associated Lymphoid Tissue) [118–121]. MALTomas may, however, express *bcl*-2 protein, confirming that it may be expressed independently of t(14;18) [122]. Similarly, *bcl*-2 involvement has not been observed in monocytoid B-cell lymphoma [123], mediastinal large B-cell lymphoma of young adults [124], primary cutaneous B-cell lymphoma [125], and in AIDS-associated lymphoma [126]. Rearrangements have been detected cytogenetically in some myelomas and Waldenstrom's macroglobulinemias [127], in salivary gland lymphomas [128], in other nonfollicle center cell lymphomas [129], and also in some T-cell-rich large B-cell lymphomas [130]. The diversity of diseases with which *bcl*-2 is associated is consistent with what is currently understood about its function. Curiously, *bcl*-2 rearrangements have been detected in benign reactive follicular hyperplasia [131,132]. Until longitudinal follow-up studies are conducted, we do not yet know whether this finding serves as a marker to predict which patients may subsequently develop overt lymphomas. Alternatively, there may be a low, but finite, number of cells in normal individuals that carry t(14;18) but that lack other, as yet undiscovered, factors needed for malignant transformation.

Progression of diseases with bcl-2 *involvement*

As noted previously, *bcl*-2 appears to function to protect cells from programmed cell death. When dysregulated, as in t(14;18) bearing follicular NHLs, a neoplastic proliferation/accumulation results. This disease may be viewed as a malignancy in evolution, in that the majority will progress into higher grade diseases, with the risk for conversion to more aggressive diffuse NHL, being approximately 44% at 5 years and 67% at 10 years from the initial diagnosis [82]. It is reasonable to presume that the t(14;18) alone is insufficient to account for this evolution and that additional genetic events must be involved. Based on gene rearrangement, DNA sequencing analysis,

and examination of tumor idiotype, it has been shown that there exists a common single cell origin for the primary and evolved disease [133,134]. However, in an evaluation of patients with follicular NHL over a period of time, while the translocated *bcl*-2 locus remained conserved in each individual, some variation in Ig gene rearrangement patterns was noted [133]. This indicates that clonal evolution, at the level of the Ig genes, can occur in some cases in the absence of histologic conversion.

Numerous secondary cytogenetic abnormalities appear during the evolution of follicular NHL carrying t(14;18) to more aggressive histologic subtypes. The most frequent abnormalities described are trisomy 7 and deletions of chromosome 6 [135,136]. Although the genes for the epidermal growth factor receptor and *myb*, respectively, have been mapped to the involved regions of chromosomes 7 and 6, the exact molecular genetic explanation for these observations remains to be resolved. The acquisition of deletions of the long arm of chromosome 13 is also associated with an accelerated clinical course [56], but here again, a molecular explanation is lacking. By contrast, there are numerous reports of *myc* gene involvement in the progression of t(14;18) lymphoma, supporting the synergy between *myc* and *bcl*-2 observed in the gene transfer and transgenic studies referred to earlier. Activation of the *myc* gene in follicular NHLs with *bcl*-2 involvement can result in conversion to both high-grade NHL [137–140], as well as to lymphoblastic leukemias [141–143]. In a cell line, SUDUL-5, established from a patient with lymphoblastic lymphoma, a novel three-way translocation appears to simultaneously inactivate the *bcl*-2 gene and activate the *myc* gene [144]. In summary, increased expression of *bcl*-2, on its own, in B cells does not appear to confer a significantly increased intrinsic proliferative activity, as evidenced by the usually indolent course of follicular NHL. If, however, the *myc* gene is also activated in these cells, due to secondary genetic events, more aggressive growth occurs. It should nevertheless be emphasized that in most instances of progression of this disease we do not know the exact molecular lesion, and the reported abnormalities affecting *myc* are the exception rather than the rule.

Defects of chromosome 2 (trisomy or dup 2p) have also been described in patients with follicular NHL, and these are associated with progression of the disease [56]. It has been demonstrated that the *rel* proto-oncogene is rearranged in this setting [145]. This oncogene is homologous to the NF-κB family of transcription factors, and provocatively, v-*rel* initiated bursal neoplasms are accompanied by the resistance to the induction of apoptosis [146]. This then suggests that the activation of two oncogenes involved in the regulation of programmed cell death may be involved in the development and/or evolution of follicular NHL.

bcl-2 in diagnosis and prognosis

Diagnosis

A variety of different strategies exist for the evaluation of *bcl*-2 involvement in human diseases. At a genetic level, increasing sensitivity for the detection of rearrangement is apparent through the spectrum of conventional cytogenetics, Southern blotting, and PCR. Pulsed-field gel electrophoresis may supercede these strategies, including PCR, in the detection of relatively small clonal cell populations [147]. The recent description of antibodies that detect the *bcl*-2 protein should also prove to be of diagnostic value.

A number of chromosome 18 specific probes can be used to analyze restriction enzyme digested tumor cell DNA for evidence of rearrangement [64,67,98]. These allow the sensitive detection of clonal rearrangements when neoplastic cells comprise only a minority of the cells present and can be found in the context of both follicular and diffuse NHL. In addition to analyzing tissue samples for *bcl*-2 rearrangement, fine needle aspirate specimens are also amenable to similar Southern analysis [148]. The clustering of breakpoints on both chromosome 18 and 14 has lent itself to the use of PCR. Specific fusion gene amplification can be performed with universal primers for the J_H genes at the 3′ end and with primers for the *mbr* [149–151] and *mcr* [66] at the 5′ end of the fusion gene. These sensitive techniques allow for the detection of as little as one lymphoma cell in 10^6 normal cells, and thus for the identification of occult/minimal residual disease. Subclinical evidence of neoplastic cells can be discerned when most other techniques are negative. The clinical significance of such sensitive detection needs to be evaluated in longitudinal trials. Indeed, circulating cells carrying the t(14;18) translocation can be detected by PCR in patients in long-term remission (up to 16 years), raising doubt regarding their presence as a possible predictor of relapse [152]. Furthermore, the description of PCR detectable fusion genes in nonneoplastic scenarios [131,132] suggests that PCR may not be that specific for discerning minimal disease. PCR amplification does, however, enable the rapid sequencing of the breakpoints, without the need for conventional and laborious cloning. PCR can also be easily performed with a mixture of *mbr* and *mcr* primers, with the products being analyzed with nonradioactive probes [153].

Despite the enhanced sensitivity of PCR, not all cases that are positive by standard Southern analysis are detectable with this amplification procedure [154]. This is probably due to the choice of primers and highlights the need for careful attention to be paid to the design of primer(s) for such analyses. Many initial diagnostic specimens may not have been prospectively processed for DNA analysis. These would be useful for the detection of a tumor-specific *bcl*-2-Ig fingerprint, for monitoring occult disease. However, it has been demonstrated that formalin-fixed tissue is amenable to analysis by PCR [58,155,156]. PCR studies can also be performed for staging pur-

poses, for the detection of occult disease in cytologically and morphologically normal tissues, such as blood, bone marrow, and other fluids [152,157–159]. More useful, however, is the role of PCR in detecting minimal disease following intensive chemotherapy [160]. In this context, PCR is able to discern disease in the marrow despite evidence of histologic remission. Similarly, PCR can be utilized for the detection of residual disease following bone marrow purging in patients undergoing autologous bone marrow transplantation [161,162]. In follow-up studies, patients whose marrows are PCR negative pretransplant remain PCR negative posttransplant. The loss of PCR-detectable lymphoma following purging is associated with a significantly increased disease-free survival when compared with those patients whose marrows harbored PCR-detectable cells [163]. All patients who relapsed posttransplantation were PCR positive; however, not all patients who were PCR positive relapsed, even after up to 88 months of follow-up. Clearly, in this situation too, longitudinal studies are required to evaluate the merit of these findings. Amplification of the fusion gene is sometimes useful in the determination of clonality and lineage, when conventional Ig gene rearrangement and surface Ig studies are negative or equivocal [164,165].

In an analysis of mRNA levels, expression of *bcl*-2 message has been found to be heterogeneous in a spectrum of hematopoietic malignancies, that did not have *bcl*-2 DNA rearrangement [166]. In T-cell malignancies, expression was higher in acute compared with chronic leukemias. Expression was undetectable in precursor B-cell acute leukemias, but *bcl*-2 message was highly expressed in 50% of plasma cell dyscrasias. Given the observation that the translocated *bcl*-2 gene in the context of t(14;18) results in higher mRNA levels, it was reasoned that the detection of the chimeric transcript using cDNA-PCR may be as, if not more, sensitive than DNA-PCR. Indeed, fusion transcripts can be discerned in primary lymphoma specimens, sometimes in the absence of detectable genomic rearrangements [88].

Immunostaining with anti-*bcl*-2 antibodies has revealed that many tumors without the t(14;18) translocation, and also many normal tissues, are *bcl*-2 protein positive [19–21,167]. This may suggest that such studies are of limited diagnostic value and certainly show that expression of the protein is not a specific marker for t(14;18) positive lymphomas, as had been proposed [18]. However, the major diagnostic utility of immunostaining may be in the differentiation of benign follicular hyperplasia from malignant follicular NHL, in which the immunolocalization of *bcl*-2 is quite distinct [20]. The staining pattern is inverted in these two situations, so that *bcl*-2 is localized to the follicular mantle cells in the reactive context, with the cells within the follicle being negative, while in the neoplastic setting the cells within the follicle are intensely positive.

Prognosis

A number of factors have been proposed to be of prognostic relevance in patients with follicular NHL [168]. It was initially thought that cytogenetics might add important prognostic information, as it does in patients with acute leukemias [169]. The data in patients with follicular NHL are conflicting, with some reports that patients with the t(14;18) have a longer survival compared with patients with other cytogenetic abnormalities [170], while other reports suggest that patients without the t(14;18) translocation respond better to therapy than those who do have this translocation [171]. Other studies, using molecular techniques to identify the translocation, conventional cytogenetics, or *bcl*-2 protein expression, showed no correlation with survival [172,173]. A t(14;18) positive de novo B-cell acute lymphoblastic leukemia was recently described, and this disease seems to have a poor prognosis [174].

In patients with diffuse NHL, no clear message has emerged from the reports on the prognostic value of *bcl*-2 rearrangements. The finding of *bcl*-2 rearrangements has been shown to be associated with a relatively poor prognosis [171] and with a significantly higher incidence of extranodal disease [175]. In other studies, while *bcl*-2 rearrangements have been shown not to affect overall survival, patients with this lesion appeared to have a longer survival in partial remission, resembling the clinical behavior of follicular NHL [176,177].

Conclusions and future directions

The unravelling of *bcl*-2 has provided significant insights in a number of areas, including normal cell biology and tumor biology. It appears to be a proto-oncogene with a unique role, functioning to extend cell survival by inhibiting programmed cell death, independent of stimulating proliferation. The phenomenon of prolonging B-cell life, as a consequence of its activation, appears to be tumorigenic, both in itself as well as by increasing the risk for secondary genetic events, particularly involving *myc* and perhaps also *rel*.

Despite the information that has emerged recently, there remain many unanswered questions. We need to learn more about the factors involved in the regulation of expression of the gene, both physiologically and pathologically. What is the biochemical basis for its function in blocking apoptosis, and how does it interact with other pathways, including the NGF receptor superfamily and the *rel* gene product?

Finally, what is the potential role for targeting *bcl*-2 in the therapy of diseases in which it is overexpressed? There is precedent for the utility of antisense-mediated inhibition of other oncogenes implicated in tumorigenesis, such as *myc* and *myb* [178,179]. Indeed, phosphodiester and

phosphorothionate antisense oligonucleotides inhibit *bcl*-2 protooncogene expression, and leukemic cell growth and survival [180]. Of concern, however, is the potential effect of inhibition of the wild-type *bcl*-2 message. Given the detection of the fusion message in primary follicular NHL cells [88], antisense oligonucleotides directed specifically at the tumor-specific fusion message may overcome this problem and selectively inhibit only the abnormal cells. A similar strategy has been employed in the context of the *bcr-abl* hybrid message seen in chronic myelogenous leukemia [181]. Another potential application would perhaps be to exploit the ability of *bcl*-2 to enhance B-cell survival and immunoglobulin production, and to heighten the response to immunization [40]. The practical utilities of this might include production of monoclonal antibodies to rare specificities and perhaps also in enhancing the response to vaccines.

References

1. Weinberg RA: Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. Cancer Res 49:3713–3721, 1989.
2. Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM: Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science 226:1097–1099, 1984.
3. Ngan B-Y, Berinstein N: Oncogene involvement in lymphoid malignancy. Tumor Biol 11(Suppl. 1):78–93, 1990.
4. Tsujimoto Y, Yunis J, Onorato-Showe L, Erikson J, Nowell PC, Croce CM: Molecular cloning of the chromosomal breakpoint of B-cell lymphomas and leukemias with the t(11;14) chromosome translocation. Science 224:1403–1406, 1984.
5. Silverman GA, Jockel JI, Domer PH, Mohr RM, Taillon-Miller P, Korsmeyer SJ: Yeast artificial chromosome cloning of a two-megabase-size contig within chromosomal band 18q21 establishes physical linkage between BCL2 and plasminogen activator inhibitor type-2. Genomics 9:219–228, 1991.
6. Seto M, Jaeger U, Hockett RD, et al.: Alternative promoters and exons, somatic mutation and deregulation of the Bcl-2-Ig fusion gene in lymphoma. EMBO J7:123–131, 1988.
7. Graninger WB, Seto M, Boutain B, Goldman P, Korsmeyer SJ: Expression of Bcl-2 and Bcl-2-Ig fusion transcripts in normal and neoplastic cells. J Clin Invest 80:1512–1515, 1987.
8. Silverman GA, Green ED, Young RL, Jockel JI, Domer PH, Korsmeyer SJ: Meiotic recombination between yeast artificial chromosomes yields a single clone containing the entire BCL2 protooncogene. Proc Natl Acad Sci USA 87:9913–9917, 1990.
9. Reed JC, Tsujimoto Y, Alpers JD, Croce CM, Nowell PC: Regulation of bcl-2 proto-oncogene expression during normal human lymphocyte proliferation. Science 236:1295–1299, 1987.
10. Reed JC, Tsujimoto Y, Epstein SF, et al.: Regulation of bcl-2 gene expression in lymphoid cell lines containing normal #18 or t(14;18) chromosomes. Oncogene Res 4:271–282, 1989.
11. Makover D, Cuddy M, Yum S, et al.: Phorbol ester-mediated inhibition of growth and regulation of proto-oncogene expression in the human T cell leukemia line Jurkat. Oncogene 6:455–460, 1991.
12. Cleary ML, Smith SD, Sklar J: Cloning and structural analysis of cDNAs for bcl-2 and a

hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell 47:19–28, 1986.
13. Tsujimoto Y, Croce CM: Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proc Natl Acad Sci USA 83:5214–5218, 1986.
14. Pearson GR, Luka J, Petti L, et al.: Identification of an Epstein-Barr virus early gene encoding a second component of the restricted early antigen complex. Virology 160:151–161, 1987.
15. Chen-Levy Z, Cleary ML: Membrane topology of the bcl-2 proto-oncogenic protein demonstrated in vitro. J Biol Chem 265:4929–4933, 1990.
16. Chen-Levy Z, Nourse J, Cleary ML: The bcl-2 candidate proto-oncogene product is a 24-kilodalton integral-membrane protein highly expressed in lymphoid cell lines and lymphomas carrying the t(14;18) translocation. Mol Cell Biol 9:701–710, 1989.
17. Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ: Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348:334–336, 1990.
18. Ngan B-Y, Chen-Levy Z, Weiss LM, Warnke RA, Cleary ML: Expression in non-Hodgkin's lymphoma of the bcl-2 protein associated with the t(14;18) chromosome translocation. N Engl J Med 318:1638–1644, 1988.
19. Pezzella F, Tse AGD, Cordell JL, Pulford KAF, Gatter KC, Mason DY: Expression of the bcl-2 oncogene protein is not specific for the 14;18 chromosomal translocation. Am J Pathol 137:225–232, 1990.
20. Zutter M, Hockenbery D, Silverman GA, Korsmeyer SJ: Immunolocalization of the Bcl-2 protein within hematopoietic neoplasms. Blood 78:1062–1068, 1991.
21. Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsmeyer SJ: BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc Natl Acad Sci USA 88:6961–6965, 1991.
22. Nunez G, Seto M, Seremetis S, et al.: Growth- and tumor-promoting effects of deregulated bcl-2 in human B-lymphoblastoid cells. Proc Natl Acad Sci USA 86:4589–4593, 1989.
23. Tsujimoto Y: Overexpression of the human BCL-2 gene product results in growth enhancement of Epstein-Barr virus immortalized B cells. Proc Natl Acad Sci USA 86:1958–1962, 1989.
24. Vaux DL, Cory S, Adams JM: Bcl-2 gene promotes hemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 335:440–442, 1988.
25. Henderson S, Rowe M, Gregory C, et al.: Induction of *bcl-2* expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell 65:1107–1115, 1991.
26. Nunez G, London L, Hockenberry D, Alexander M, McKearn JP, Korsmeyer SJ: Deregulated bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. J Immunol 144:3602–3610, 1990.
27. Tsujimoto Y: Stress-resistance conferred by high level of bcl-2α protein in human B lymphoblastoid cell. Oncogene 4:1331–1336, 1989.
28. Haldar S, Reed JC, Beatty C, Croce CM: Role of bcl-2 in growth factor triggered signal transduction. Cancer Res 50:7399–7401, 1990.
29. Haldar S, Beatty C, Tsujimoto Y, Croce CM: The bcl-2 gene encodes a novel G protein. Nature 342:195–198, 1989.
30. Monica K, Chen-Levy Z, Cleary ML: Small G proteins are expressed ubiquitously in lymphoid cells and do not correspond to Bcl-2. Nature 346:189–191, 1990.
31. Reed J, Haldar S, Cuddy MP, Croce C, Makover D: Deregulated BCL2 expression enhances growth of a human B cell line. Oncogene 4:1123–1127, 1989.
32. Reed JC, Cuddy M, Slabiak T, Croce CM, Nowell PC: Oncogenic potential of bcl-2 demonstrated by gene transfer. Nature 336:259–261, 1988.
33. Reed J, Haldar S, Croce CM, Cuddy MP: Complementation by BCL2 and C-HA-RAS

oncogenes in malignant transformation of rat embryo fibroblasts. Mol Cell Biol 10:4370–4374, 1990.
34. Reed JC, Cuddy M, Haldar S, et al.: BCL-2-mediated tumorigenicity of a human T-lymphoid cell line: Synergy with MYC and inhibition by BCL-2 antisense. Proc Natl Acad Sci USA 87:3660–3664, 1990.
35. McDonnell TJ, Deane N, Platt FM, et al.: Bcl-2-Immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell 57:79–88, 1989.
36. McDonnell TJ, Nunez G, Platt FM, et al.: Deregulated bcl-2-immunoglobulin transgene expands a resting but responsive immunoglobulin M and D-expressing B-cell population. Mol Cell Biol 10:1901–1907, 1990.
37. Nunez G, Hockenbery D, McDonnell TJ, Sorensen CM, Korsmeyer SJ: Bcl-2 maintains B cell memory. Nature 353:71–73, 1991.
38. McDonnell TJ, Korsmeyer SJ: Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14;18). Nature 349:254–256, 1991.
39. Strasser A, Harris AW, Vaux DL, et al.: Abnormalities of the immune system induced by dysregulated bcl-2 expression in transgenic mice. Curr Top Microbiol Immunol 166:175–181, 1990.
40. Strasser A, Whittingham S, Vaux DL, et al.: Enforced *BCL2* expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. Proc Natl Acad Sci USA 88:8661–8665, 1991.
41. Strasser A, Harris AW, Bath ML, Cory S: Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature 348:331–333, 1990.
42. Arends MJ, Wyllie AH: Apoptosis: Mechanisms and roles in pathology. Int Rev Exp Pathol 32:223–254, 1991.
43. Williams GT: Programmed cell death: Apoptosis and oncogenesis. Cell 65:1097–1098, 1991.
44. Liu YJ, Mason DY, Johnson GD, et al.: Germinal center cells express bcl-2 protein after activation by signals which prevent their entry into apoptosis. Eur J Immunol 1991:1905–1910, 1991.
45. Liu YJ, Joshua DE, Williams GT, Smith CA, Gordon J, MacLennan IC: Mechanism of antigen-driven selection in germinal centres. Nature 342:929–931, 1989.
46. Hollowood K, Macartney JC: Reduced apoptotic cell death in follicular lymphoma. J Pathol 163:337–342, 1991.
47. Mallett S, Barclay AN: A new superfamily of cell surface proteins related to the nerve growth factor receptor. Immunol Today 12:220–223, 1991.
48. Trauth BC, Klas C, Peters AMJ, et al.: Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science 245:301–305, 1989.
49. Itoh N, Yonehara S, Ishii A, et al.: The polypeptide encoded by the cDNA for human cell surface antigen fas can mediate apoptosis. Cell 66:233–243, 1991.
50. Laster SM, Wood JG, Gooding LR: Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. J Immunol 141:2629–2634, 1988.
51. Ryffel B, Brockhaus M, Durmuller U, Gudat F: Tumor necrosis factor receptors in lymphoid tissues and lymphomas. Source and site of action of tumor necrosis factor alpha. Am J Pathol 139:7–15, 1991.
52. Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M: Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature 352:345–349, 1991.
53. Prokocimer M, Shaklai M, Bassat HB, Wolf D, Godfinger N, Rotter V: Expression of p53 in human leukemia and lymphoma. Blood 68:113–118, 1986.
54. Owens GP, Hahn WE, Cohen JJ: Identification of mRNAs associated with programmed cell death in immature thymocytes. Mol Cell Biol 11:4177–4188, 1991.
55. Mangeney M, Richard Y, Coulaud D, Tursz T, Wiels J: CD77: An antigen of germinal center B cells entering apoptosis. Eur J Immunol 21:1131–1140, 1991.

56. Yunis JJ, Frizzera G, Oken MM, McKenna J, Theoliogides A, Arnesen M: Multiple recurrent genomic defects in follicular lymphoma. A possible model for cancer. N Engl J Med 316:79–84, 1987.
57. Tsujimoto Y, Ikegaki N, Croce CM: Characterization of the protein product of bcl-2, the gene involved in human follicular lymphoma. Oncogene 2:3–7, 1987.
58. Pezzella F, Ralfkiaer E, Gatter KC, Mason DY: The 14;18 translocation in European cases of follicular lymphoma: Comparison of Southern blotting and the polymerase chain reaction. Br J Haematol 76:58–64, 1990.
59. Amakawa R, Fukuhara S, Ohno H, et al.: Involvement of bcl-2 gene in Japanese follicular lymphoma. Blood 73:787–791, 1989.
60. Liang R, Chan V, Chan TK, Chiu E, Todd D: Rearrangement of immunoglobulin, T-cell receptor, and bcl-2 genes in malignant lymphomas in Hong Kong. Cancer 66:1743–1747, 1990.
61. Loke SL, Pittaluga S, Srivastava G, Raffeld M, Ho FCS: Translocation of bcl-2 gene in non-Hodgkin's lymphoma in Hong Kong Chinese. Br J Haematol 76:65–69, 1990.
62. Bakhshi A, Jensen JP, Goldman P, et al.: Cloning of the chromosomal breakpoint of t(14;18) human lymphomas: Clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell 41:899–906, 1985.
63. Cleary ML, Sklar J: Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc Natl Acad Sci USA 82:7439–7443, 1985.
64. Lipford E, Wright JJ, Urba W, et al.: Refinement of lymphoma cytogenetics by the chromosome 18q21 major breakpoint region. Blood 70:1816–1823, 1987.
65. Cleary ML, Galili N, Sklar J: Detection of a second t(14;18) breakpoint cluster region in human follicular lymphoma. J Exp Med 164:315–320, 1986.
66. Ngan B-Y, Nourse J, Cleary ML: Detection of chromosomal translocation t(14;18) within the minor cluster region of bcl-2 by polymerase chain reaction and direct genomic sequencing of the enzymatically amplified DNA in follicular lymphomas. Blood 73:1759–1762, 1989.
67. Weiss LM, Warnke RA, Sklar J, Cleary ML: Molecular analysis of the t(14;18) chromosome translocation in malignant lymphomas. N Engl J Med 317:1185–1189, 1987.
68. Tsujimoto Y, Bashir MM, Givol I, Cossman J, Jaffe E, Croce CM: DNA rearrangements in follicular lymphoma can involve the 5′ or 3′ region of the bcl-2 gene. Proc Natl Acad Sci USA 84:1329–1331, 1987.
69. Mikraki V, Ladanyi M, Chaganti RSK: Structural alterations in the 5′ region of the BCL2 gene in follicular lymphomas with BCL2-MBR or BCL2-MCR rearrangements. Genes Chromosomes Cancer 3:117–121, 1991.
70. Cotter F, Price C, Zucca E, Young BD: Direct sequence analysis of the $14q^+$ and $18q^-$ chromosome junctions in follicular lymphoma. Blood 76:131–135, 1990.
71. Kneba M, Eick S, Herbst H, et al.: Frequency and structure of t(14;18) major breakpoint regions in non-Hodgkin's lymphomas typed according to the Kiel classification: Analysis by direct DNA sequencing. Cancer Res 51:3243–3250, 1991.
72. Hillion J, Mecucci C, Aventin A, et al.: A variant translocation t(2;18) in follicular lymphoma involves the 5′ end of bcl-2 and Igκ light chain gene. Oncogene 6:169–172, 1991.
73. Osada H, Seto M, Ueda R, et al.: *bcl-2* Gene rearrangement analysis in Japanese B cell lymphoma; Novel *bcl-2* recombination with immunoglobulin κ chain gene. Jpn J Cancer Res 80:711–715, 1989.
74. Larsen CJ, Mecucci C, Leroux D: t(2;18) and t(18;22) variant chromosomal translocations and bcl-2 gene rearrangements in human malignant lymphomas. Nouv Rev Fr Hematol 32:401–403, 1990.
75. Leroux D, Hillion J, Monteil M, et al.: t(18;22)(q21;q11) with rearrangement of *bcl-2* as a

possible secondary change in a lymphocytic lymphoma. Genes Chromosomes Cancer 3:205–209, 1991.
76. Seto M, Osada H, Ueda R, et al.: bcl-2 translocation in Japanese B cell lymphoma: Novel bcl-2 translocation with immunoglobulin heavy chain diversity segment. Jpn J Cancer Res 82:65–71, 1991.
77. Bakhshi A, Wright JJ, Graninger W, et al.: Mechanism of the t(14;18) chromosomal translocation: Structural analysis of both derivative 14 and 18 reciprocal partners. Proc Natl Acad Sci USA 84:2396–2400, 1987.
78. Tsujimoto Y, Gorham J, Cossman J, Jaffe E, Croce CM: The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. Science 229:1390–1393, 1985.
79. Tycko B, Sklar J: Chromosomal translocations in lymphoid neoplasia: A reappraisal of the recombinase model. Cancer Cells 2:1–8, 1990.
80. Bertoli LF, Kubagawa H, Borzillo GV, et al.: Bone marrow origin of a B-cell lymphoma. Blood 72:94–101, 1988.
81. Turka LA, Schatz DG, Dettinger MA, et al.: Thymocyte expression of RAG-1 and RAG-2 — termination by T-cell receptor cross-linking. Science 253:778–781, 1991.
82. Zelenetz AD, Campbell MJ, Bahler DW, et al.: Follicular lymphoma: A model of lymphoid tumor progression in man. Ann Oncol 2(Suppl. 2):115–122, 1991.
83. Tsujimoto Y, Cossman J, Jaffe E, Croce CM: Involvement of the bcl-2 gene in human follicular lymphoma. Science 228:1440–1443, 1985.
84. Krowczynska AM, Rudders RA, Krontiris TG: The human minisatellite consensus at breakpoints of oncogene translocations. Nucleic Acids Res 18:1121–1127, 1990.
85. Wyatt RT, Rudders RA, Dellelis RA, Krontiris TG: Site, sequence and potential haplotype specificity of bcl2 translocation. Blood 76(Suppl. 1):379a, 1990.
86. Adachi M, Tsujimoto Y: Potential Z-DNA elements surround the breakpoints of chromosome translocation within the 5′ flanking region of bcl-2 gene. Oncogene 5:1653–1657, 1990.
87. Hua C, Zorn S, Jensen JP, et al.: Consequences of the t(14;18) chromosomal translocation in follicular lymphoma: Deregulated expression of a chimeric and mutated BCL-2 gene. Oncogene Res 2:263–275, 1988.
88. Bagg A, Stetler-Stevenson M, Hedvat C, Irving S, Cossman J: Detection of chimeric bcl-2/IgH transcripts in primary human lymphoma. Blood 76(Suppl. 1):341a, 1990.
89. Bohjanen PR, Petryniak B, June CH, Thompson CB, Lindsten T: An inducible cytoplasmic factor (AU-B) binds selectively to AUUUA multimers in the 3′ untranslated region of lymphokine mRNA. Mol Cell Biol 11:3288–3295, 1991.
90. Brewer G: An A+U-rich element RNA-binding factor regulates c-myc mRNA stability in vitro. Mol Cell Biol 11:2460–2466, 1991.
91. Shaw G, Kamen R: A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46:659–667, 1986.
92. Ross HJ, Sato N, Ueyama Y, Koeffler HP: Cytokine messenger RNA stability is enhanced in tumor cells. Blood 77:1787–1795, 1991.
93. Harrold S, Genovese C, Kobrin B, Morrison SL, Milcarek C: A comparison of apparent mRNA half-life using kinetic labelling techniques vs. decay following administration of transcriptional inhibitors. Anal Biochem 198:19–29, 1991.
94. Arrick BA, Lee AL, Grendell RL, Derynck R: Inhibition of translation of transforming growth factor-β3 mRNA by its untranslated region. Mol Cell Biol 11:4306–4313, 1991.
95. Ch'ng JL, Shoemaker DL, Schimmel P, Holmes EW: Reversal of creatine kinase translational repression by 3′ untranslated sequences. Science 248:1003–1006, 1990.
96. Hua C, Raffeld M, Ko H-S, Fast P, Bakhshi A, Cossman J: Mechanism of bcl-2 activation in human follicular lymphoma. Oncogene 5:233–235, 1990.
97. Tanaka S, Kant J, Reed JC: G to A polymorphism in the second exon of the BCL2 gene. Nucleic Acids Res 19:1964, 1991.

98. Aisenberg AC, Wilkes BM, Jacobson JO: The bcl-2 gene is rearranged in many diffuse B-cell lymphomas. Blood 71:969–972, 1988.
99. Medeiros LJ, Lardelli P, Stetler-Stevenson M, Longo DL, Jaffe ES: Genotypic analysis of diffuse, mixed cell lymphomas. Am J Clin Pathol 95:547–555, 1991.
100. Durie BGM, Mason DY, Giles F, et al.: Expression of the bcl-2 oncogene protein in multiple myeloma. Blood 76(Suppl. 1):347a, 1990.
101. Adachi M, Cossman J, Longo D, Croce CM, Tsujimoto Y: Variant translocation of the bcl-2 gene to immunoglobulin λ light chain gene in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 86:2771–2774, 1989.
102. Adachi M, Tsujimoto Y: Juxtaposition of human *bcl-2* and immunoglobulin lambda light chain gene in chronic lymphocytic leukemia is the result of a reciprocal chromosome. Oncogene 4:1073–1075, 1989.
103. Adachi M, Tefferi A, Griepp PR, Kipps TJ, Tsujimoto Y: Preferential linkage of bcl-2 to immunoglobulin light chain gene in chronic lymphocytic leukemia. J Exp Med 171:559–564, 1990.
104. Raghoebier S, van Krieken JHJM, Kluin-Nelemans JC, et al.: Oncogene rearrangements in chronic B-cell leukemia. Blood 77:1560–1564, 1991.
105. Levine EG, Arthur DC, Machnicki J, et al.: Four new recurring translocations in non-Hodgkin's lymphoma. Blood 74:1796–1800, 1989.
106. Stetler-Stevenson M, Crush-Stanton S, Cossman J: Involvement of the bcl-2 gene in Hodgkin's disease. J Natl Cancer Inst 82:855–858, 1990.
107. Cabanillas F, Pathak S, Trujillo J, et al.: Cytogenetic features of Hodgkin's disease suggest possible origin from a lymphocyte. Blood 71:1615–1617, 1988.
108. Reid A, Frizzera G, O'Leary TJ: Detection of the t(14;18) translocation in Hodgkin's disease (HD) by polymerase chain reaction. Lab Invest 64:83A, 1991.
109. Masih A, Nakamine H, Wickert R, Mitchell D, Weisenberger D: Molecular genetic analysis of extranodal non-Hodgkin's lymphoma. Lab Invest 64:77A, 1991.
110. Gupta RK, Thompson IW, Norton AJ, Young BD, Bodmer JG, Lister TA: The t(14;18) chromosomal translocation and bcl-2 protein expression in Hodgkin's disease. Blood 78 (Suppl. 1), 1991.
111. Athan E, Chadburn A, Knowles DM: The bcl-2 gene translocation is infrequently detected in Hodgkin's disease by Southern blot hybridization and polymerase chain reaction. Lab Invest 64:64A, 1991.
112. Said JW, Sassoon AF, Shintaku IP, Kurtin PJ, Pinkus GS: Absence of bcl-2 major breakpoint region and J_H gene rearrangement in lymphocyte predominance Hodgkin's disease. Results of Southern blot analysis and polymerase chain reaction. Am J Pathol 138:261–264, 1991.
113. Mueller N: An epidemiologist's view of the new molecular biology findings in Hodgkin's disease. Ann Oncol 2:23–28, 1991.
114. Knecht H, Odermatt BF, Bachmann E, et al.: Frequent detection of Epstein-Barr virus DNA by the polymerase chain reaction in lymph node biopsies from patients with Hodgkin's disease without genomic evidence of B- or T-cell clonality. Blood 78:760–767, 1991.
115. Brousset P, Chittal S, Schlaifer D, et al.: Detection of Epstein-Barr virus messenger RNA in Reed-Sternberg cells of Hodgkin's disease by in situ hybridization with biotinylated probes on specially processed modified acetone methyl benzoate xylene (ModAMeX) sections. Blood 77:1781–1786, 1991.
116. Pallesen G, Hamilton-Dutoit SJ, Rowe M, Young LS: Expression of Epstein-Barr virus latent gene products in tumour cells of Hodgkin's disease. Lancet 337:320–322, 1991.
117. Herbst H, Dallenbach F, Hummel M, et al.: Epstein-Barr virus latent membrane protein expression in Hodgkin and Reed-Sternberg cells. Proc Natl Acad Sci USA 88:4766–4770, 1991.
118. van Krieken JHJM, Raffeld M, Raghoebier S, Jaffe ES, van Ommen GJB, Kluin PM: Molecular genetics of gastrointestinal non-Hodgkin's lymphomas: Unusual prevalence and

pattern of c-myc rearrangements in aggressive lymphomas. Blood 76:797–800, 1990.
119. Pan L, Diss TC, Cunningham D, Isaacson PG: The bcl-2 gene in primary B cell lymphoma of mucosa-associated lymphoid tissue (MALT). Am J Pathol 135:7–11, 1989.
120. Knowles DM, Athan E, Ubriaco A, et al.: Extranodal noncutaneous lymphoid hyperplasias representa continuous spectrum of B-cell neoplasia: Demonstration by molecular genetic analysis. Blood 73:1635–1645, 1989.
121. Clark H, Jones DB, Jacobs P, Wright DH: Molecular and cytogenetic analysis of t(14;18) in nodal and extranodal lymphoma. J Pathol 161:340A, 1990.
122. Isaacson PG, Wotherspoon AC, Diss TC, Pan LX: *bcl-2* expression in lymphomas. Lancet 337:175–176, 1991.
123. Ngan B-Y, Warnke RA, Wilson M, Takagi K, Cleary ML, Dorfman RF: Monocytoid B-cell lymphoma: A study of 36 cases. Human Pathol 22:409–421, 1991.
124. Scarpa A, Borgato L, Chilosi M, et al.: Evidence of *c-myc* gene abnormalities in mediastinal large B-cell lymphoma of young adult age. Blood 78:780–788, 1991.
125. Delia D, Borrello MG, Berti E, et al.: Clonal immunoglobulin gene rearrangements and normal T-cell receptor, bcl-2, and c-myc genes in primary cutaneous B-cell lymphomas. Cancer Res 49:4901–4905, 1989.
126. Subar M, Neri A, Inghirami G, Knowles DM, Dalla-Favera R: Frequent c-myc oncogene activation and infrequent presence of Epstein-Barr virus genome in AIDS-associated lymphoma. Blood 72:667–671, 1988.
127. Nishida K, Taniwaka M, Misawa S, Abe T: Nonrandom rearrangement of chromosome 14 at band q32.33 in human lymphoid malignancies with mature B-cell phenotype. Cancer Res 49:1275–1281, 1989.
128. Kerrigan DP, Irons J, Chen I-M: Bcl-2 gene rearrangement in salivary gland lymphoma. Am J Surg Pathol 14:1133–1138, 1990.
129. Vandenberghe E, de Wolf-Peeters C, Delabie J, et al.: Cytogenetic characterization of three cases of unusual B-cell non-Hodgkin's lymphoma. Cancer Genet Cytogenet 53:229–235, 1991.
130. Krishnan J, Ventre K, Reid A, O'Leary T, Frizzera G: T cell rich large B cell lymphoma: A study of clinical, morphologic and immunohistochemical features, and PCR analysis of bcl-2 rearrangements. Lab Invest 64:76A, 1991.
131. Braziel R, Grogan T, Berman M, et al.: Bcl-2/JH translocation in follicular lymphoid hyperplasia. Lab Invest 64:68A, 1991.
132. Limpens J, de Jong D, Voetdijk AMH, et al.: Translocation t(14;18) in benign lymphocytes. Blood 76(Suppl. 1):237a, 1990.
133. Raffeld M, Wright JJ, Lipford E, et al.: Clonal evolution of t(14;18) follicular lymphomas demonstrated by immunoglobulin genes and the 18q21 major breakpoint region. Cancer Res 47:2537–2542, 1987.
134. Zelenetz AD, Chen TT, Levy R: Histologic transformation of follicular lymphoma to diffuse lymphoma represents tumor progression by a single malignant B cell. J Exp Med 173:197–207, 1991.
135. Richardson ME, Quanguang C, Filippa DA, et al.: Intermediate- to high-grade histology of lymphomas carrying t(14;18) is associated with additional nonrandom chromosome changes. Blood 70:444–447, 1987.
136. Armitage JO, Sanger WG, Weisenburger DD, et al.: Correlation of secondary cytogenetic abnormalities with histologic appearance in non-Hodgkin's lymphomas bearing t(14;18) (q32;q21). J Natl Cancer Inst 80:576–580, 1988.
137. Lee JT, Innes DJ, Williams ME: Sequential bcl-2 and c-myc oncogene rearrangements associated with the clinical transformation of non-Hodgkin's lymphoma. J Clin Invest 84:1454–1459, 1989.
138. Koduru PRK, Offit K: Molecular structure of double reciprocal translocations: Significance in B-cell lymphomagenesis. Oncogene 6:145–148, 1991.
139. De Jong D, Voetdijk BMH, Beverstock GC, van Ommen GJB, Willemze R, Kluin PM:

Activation of the c-myc oncogene in a precursor-B-cell blast crises of follicular lymphoma, presenting as composite lymphoma. N Engl J Med 318:1373–1378, 1988.
140. Aventin A, Mecucci C, Guanyabens C, et al.: Variant t(2;18) translocation in a Burkitt conversion of follicular lymphoma. Br J Haematol 74:367–369, 1990.
141. Brito-Babapulle V, Crawford A, Khokhar T, et al.: Translocations t(14;18) and t(8;14) with rearranged *bcl-2* and *c-myc* in a case presenting as B-ALL (L3). Leukemia 5:83–87, 1991.
142. Gauwerky CE, Haluska FG, Tsujimoto Y, Nowell PC, Croce CM: Evolution of B-cell malignancy: Pre-B-cell leukemia resulting from MYC activation in a B-cell neoplasm with a rearranged BCL2 gene. Proc Natl Acad Sci USA 85:8548–8552, 1988.
143. Matsuzaki H, Hata H, Asou N, et al.: Establishment and characterization of acute B-cell lymphocytic leukemia cell line showing (8;14) and (14;18) chromosome translocation. Acta Haematol 84:156–161, 1990.
144. Kiem HP, Nourse J, Saltman DL, Blume KG, Cleary ML: Concurrent activation of c-myc and inactivation of bcl-2 by chromosomal translocation in a lymphoblastic lymphoma cell line. Oncogene 5:1815–1819, 1990.
145. Lu D, Thompson JD, Gorski GK, Rice NR, Mayer MG, Yunis J: Alterations at the *rel* locus in human lymphoma. Oncogene 6:1235–1241, 1991.
146. Neiman PE, Thomas SJ, Loring G: Induction of apoptosis during normal and neoplastic B-cell development in the bursa of Fabricius. Proc Natl Acad Sci USA 88:5857–5861, 1991.
147. Zelenetz AD, Chu G, Galili N, et al.: Enhanced detection of the t(14;18) translocation in malignant lymphomas using pulsed-field gel electrophoresis. Blood 78:1552–1560, 1991.
148. Williams ME, Frierson HF, Tabbarah S, Ennis PS: Fine-needle aspiration of non-Hodgkin's lymphoma. Southern blot analysis for antigen receptor, bcl-2, and c-myc rearrangement. Am J Clin Pathol 93:754–759, 1990.
149. Stetler-Stevenson M, Raffeld M, Cohen P, Cossman J: Detection of occult follicular lymphoma by specific DNA amplification. Blood 72:1822–1825, 1988.
150. Lee M-S, Chang K-S, Cabanillas F, Freireich EJ, Trujillo JM, Stass SA: Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification. Science 237:175–178, 1987.
151. Crescenzi M, Seto M, Herzig GP, Weiss PD, Griffith RC, Korsmeyer SJ: Thermostable DNA polymerase chain amplification of t(14;18) chromosome breakpoints and detection of minimal residual disease. Proc Natl Acad Sci USA 85:4869–4873, 1988.
152. Price CGA, Meerabux J, Murtagh S, et al.: The significance of circulating cells carrying t(14;18) in long remission from follicular lymphoma. J Clin Oncol 9:1527–1532, 1991.
153. Said JW, Sassoon AF, Shintaku IP, Corcoran P, Nichols SW: Polymerase chain reaction for bcl-2 in diagnostic lymph node biopsies. Mod Pathol 3:659–663, 1990.
154. Masih A, Sun J, Dicke K, Weisenburger D, Armitage J, Wu K: Polymerase chain reaction detection of t(14;18) involving the bcl-2 major breakpoint region: Correlation with Southern analysis. Lab Invest 64:78A, 1991.
155. Shibata D, Hu E, Weiss LM, Brynes RK, Nathwani BN: Detection of specific t(14;18) chromosomal translocations in fixed tissue. Human Pathol 21:199–203, 1990.
156. Stoler M, Fishleder A, Tubbs R, Reihsaus E, Kawasaki E: A comparison between fresh and fixed lymph node biopsies for the detection of t(14;18) translocation by polymerase chain reaction. Lab Invest 64:84A, 1991.
157. Price CGA, Cotter FE, Curling OM, et al.: Polymerase chain reaction to confirm extranodal progression of follicular lymphoma. Lancet 1:1132, 1989.
158. Price CGA, Tuszynski A, Watt SM, Murdoch SJ, Lister TA, Young BD: Detection of additional t(14;18) translocations in follicular lymphoma by PCR and direct sequencing. Proc Am Acad Cancer Res 32:292, 1991.
159. Cunningham D, Hickish T, Rosin RD, et al.: Polymerase chain reaction for detection of dissemination in gastric lymphoma. Lancet 1:695–697, 1989.
160. Gribben JG, Freedman AS, Woo SD, et al.: All advanced stage non-Hodgkin's

lymphomas with a polymerase chain reaction amplifiable breakpoint of bcl-2 have residual cells containing the bcl-2 rearrangement at evaluation and following treatment. Blood, in press, 1991.
161. Gribben JG, Blake K, Nadler LM: PCR amplification of bcl-2 translocations in the detection of minimal disease after autologous bone marrow transplantation in non Hodgkin's lymphoma. Br J Haematol 77(Suppl. 1):51, 1991.
162. Negrin RS, Kiem H-P, Schmidt-Wolf IGH, Blume KG, Cleary MC: Use of the polymerase chain reaction to monitor effectiveness of ex vivo tumor cell purging. Blood 77:654–660, 1991.
163. Gribben JG, Freedman AS, Neuberg D, et al.: Immunologic purging of polymerase chain reaction detectable lymphoma cells results in increased disease-free survival following autologous bone marrow transplantation for B-cell non-Hodgkin's lymphomas. N Engl J Med, in press, 1991.
164. Ngan B, Warnke RA, Cleary ML: Variability of immunoglobulin expression in follicular lymphoma. An immunohistologic and molecular genetic study. Am J Pathol 135:1139–1144, 1989.
165. Lardelli P, Swaby RF, Medeiros LJ, Jaffe ES, Rizzi R, Stetler-Stevenson M: Determination of lineage and clonality in diffuse lymphomas using the polymerase chain reaction technique. Human Pathol 22:685–689, 1991.
166. Mariano MT, Moretti L, Donelli A, Grantini M: Bcl-2 gene expression in hematopoietic cell differentiation. Blood 76(Suppl. 1): 105a, 1990.
167. Pezzella F, Gatter KC, Mason DY, et al.: Bcl-2 protein expression in follicular lymphomas in absence of 14;18 translocation. Lancet 336:1510–1511, 1990.
168. Bastion Y, Berger F, Bryon P-A, Felman P, French M, Coiffier B: Follicular lymphomas: Assessment of prognosis factors in 127 patients followed for 10 years. Ann Oncol 2:123–129, 1991.
169. Levine EG, Arthur DC, Frizzera G, Peterson BA, Hurd DD, Bloomfield CD: There are differences in cytogenetic abnormalities among histologic subtypes of the non-Hodgkin's lymphomas. Blood 66:1414–1422, 1985.
170. Kaneko Y, Rowley JD, Varikojis D, et al.: Prognostic implications of karyotype and morphology in patients with non-Hodgkin's lymphoma. Int J Cancer 32:683–692, 1983.
171. Yunis JJ, Mayer MG, Arnesen MA, Aeppli DP, Oken MM, Frizzera G: bcl-2 and other genomic alterations in the prognosis of large-cell lymphoma. N Engl J Med 320:1047–1054, 1989.
172. Pezzella F, Mason DY: The bcl-2 gene and 14;18 translocation in lymphoproliferative orders. Nouv Rev Fr Hematol 32:397–399, 1990.
173. Levine EG, Arthur DC, Frizzera G, Peterson BA, Hurd DD, Bloomfield CD: Cytogenetic abnormalities predict clinical outcome in non-Hodgkin's lymphoma. Ann Intern Med 108:14–20, 1988.
174. Kramer MHH, Rhagoebier S, de Jong D, Willemze R, Kluin PM, Kluin-Nelemans JC: De novo acute B-cell leukemia with translocation t(14;18): an entity with a poor prognosis. Blood 76(Suppl. 1):291a, 1990.
175. Lee M-S, Blick MB, Pathak S, et al.: The gene located at chromosome 18 band q21 is rearranged in uncultured diffuse lymphomas as well as follicular lymphomas. Blood 70:90–95, 1987.
176. Offit K, Koduru PRK, Hollis R, et al.: 18q21 rearrangement in diffuse large cell lymphoma: Incidence and clinical significance. Br J Haematol 72:178–183, 1989.
177. Offit K, Wong G, Filippa DA, Tao Y, Chaganti RSK: Cytogenetic analysis of 434 consecutively ascertained specimens of non-Hodgkin's lymphoma: Clinical correlations. Blood 77:1508–1515, 1991.
178. Sims R, Calabretta B, Gewirtz A: An in vitro model of bone marrow purging with antisense oligodeoxynucleotides to proto-oncogene C-MYB. Blood 74(Suppl. 1):19a, 1989.
179. Holt JT, Redner RL, Nienhuis AW: An oligomer complimentary to *c-myc* mRNA inhibits

proliferation of HL-60 promyelocytic cells and induces differentiation. Mol Cell Biol 8:963–973, 1988.
180. Reed JC, Stein C, Subasinghe C, et al.: Antisense-mediated inhibition of BCL2 protooncogene expression and leukemic cell growth and survival: Comparison of phosphodiester and phosphorothionate oligodeoxynucleotides. Cancer Res 50:6565–6570, 1990.
181. Szczylik C, Skorski T, Nicolaides NC, et al.: Selective inhibition of leukemia cell proliferation by BCR-ABL antisense oligodeoxynucleotides. Science 253:562–565, 1991.

8. Malignant transformation by *abl* and *BCR/ABL*

Richard A. Van Etten

Introduction

Activated *abl* oncogenes have been implicated in causing pre-B lymphoma in mice via Abelson murine leukemia virus, fibrosarcoma in cats via Hardy-Zuckerman feline sarcoma virus II, and, through the Philadelphia chromosome, are linked with the myeloproliferative syndrome chronic myelogenous leukemia and with acute lymphoid and myeloid leukemia in humans. This review will outline the current state of knowledge concerning this important oncogene family, emphasizing recent advances in our understanding.

Viral forms of *abl*

The *abl* oncogene was originally identified as the transforming gene of an acutely oncogenic murine retrovirus, Abelson murine leukemia virus (Ab-MuLV) [for review, see 1]. Ab-MuLV was isolated from a corticosteroid-treated mouse infected with Moloney murine leukemia virus (Mo-MuLV), which subsequently developed, after an unusually short latent period, a nonthymic lymphoma distinct from the thymic lymphoma characteristically induced by Mo-MuLV [2]. Mice inoculated with filtrates from tumor tissue of this animal developed an identical syndrome, confirming the infectious nature of the agent involved. For several years, the nature of the infecting virus that induced these nonthymic lymphomas (also referred to as Abelson disease) was obscure, since antisera directed against Mo-MuLV neutralized Ab-MuLV, and very dilute preparations of the infectious agent induced thymic lymphoma indistinguishable from that induced by Mo-MuLV. A major advance came when it was discovered that the infectious agent in Abelson disease was able to transform NIH3T3 fibroblasts, a property not shared by Mo-MuLV [3]. This discovery led to the recognition that the original infectious agent isolated by Abelson and Rabstein was actually a complex of replication-competent Mo-MuLV helper virus with a replication-defective derivative of Moloney virus, which we now denote Ab-MuLV. It is this defective retrovirus that, in the absence of helper virus, can induce

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Abelson lymphoma and transform 3T3 fibroblasts [4], although production of infectious virus from the transformed cells requires superinfection with Mo-MuLV helper virus.

Ab-MuLV induces a lymphoma of B lymphocytes after intraperitoneal inoculation of neonatal mice, in distinction to the T lymphoma induced by Mo-MuLV. Pathologically, the disease presents in neonates with a characteristic 'caput' or bulging of the skull, a reflection of the infiltration of the meninges and the marrow of the calvaria with malignant lymphoblasts. While virtually all strains of neonatal mice are susceptible to Ab-MuLV, there are genetic restrictions on the susceptibility of adult mice to the virus. Resistance to development of Abelson disease in adult mice appears to be immunologically mediated and segregates as two independent autosomal loci where sensitivity is dominant over resistance [5]. The only commonly used inbred mouse strain carrying sensitivity alleles at both loci is Balb/c, and adult Balb/c mice develop Abelson lymphoma with a particular propensity to involve the spinal canal and cause hind-limb paralysis after inoculation with Ab-MuLV.

The target cell for the induction of Abelson lymphoma appears to be an early B-lymphoid progenitor that is present in murine bone marrow at low numbers [6]. The malignant cells that are isolated from diseased animals have the characteristics of B-lymphocytes that are, to a first approximation, arrested at the pre-B stage of B-cell development [7]. Most Ab-MuLV-transformed B-lymphoid cell clones have carried out DJ or VDJ rearrangement at one or both alleles of the immunoglobulin heavy chain (IgH) gene locus, and some express cytoplasmic μ protein; all express the B-lymphoid cell surface antigen B220. The tumors that develop in Ab-MuLV-infected mice are usually monoclonal or oligoclonal with respect to both proviral integration site and IgH gene rearrangements, suggesting that additional genetic events must occur subsequent to viral infection in order for a malignancy to result. Ab-MuLV can transform this same cell type in vitro, yielding bone marrow-derived B-lymphoblastoid cell lines that are tumorigenic in syngeneic mice [8]. The type of malignancy that results after Ab-MuLV infection can be modulated by pretreatment of mice with pristane oil [9]. Such mice subsequently develop plasmacytomas, representative of the most mature B-lineage cells, with rearranged heavy and light chain Ig genes and expression of Ig. These tumors carry the Ab-MuLV provirus and also have rearrangements of the c-*myc* locus. Under defined conditions, Ab-MuLV can also transform a wide variety of primary hematopoietic cells, including macrophages [10], mast cells [11], and T-lymphoid cells [12]. Ab-MuLV infection of fetal liver or yolk sac cells, followed by plating in methylcellulose, yields factor-independent erythroid [13] and myeloid [14] colonies, some of which can be expanded into continuous cell lines. In addition, Ab-MuLV infection can abrogate growth factor dependence in several factor-dependent lymphoid [15,16] and myeloid [17] cell lines in vitro by a mechanism that does not appear to involve autocrine growth

factor production. These experiments indicate that the transforming ability of Ab-MuLV is wider than would be appreciated from the narrow range of disease observed after intraperitoneal inoculation.

Studies of viral RNA and protein showed that Ab-MuLV-infected cells contained a novel protein with sequences derived from the retroviral gag gene as well as sequences that appeared not to be derived from Mo-MuLV [18,19]. Tumor regressor sera, which recognized the non-Moloney portion of the novel protein, were found to crossreact with a polypeptide in normal cells of molecular weight 150 kDa, denoted normal cell protein 150 (NCP 150) [20]. Molecular cloning of the Ab-MuLV genome showed that the virus was derived from a recombination between Mo-MuLV and a normal cellular gene denoted c-*abl* [21,22]. Thus, the parental virus transduced a cellular proto-oncogene to become an acutely transforming virus, a now-familiar theme in retrovirus and oncogene research [23]. The transforming gene of Ab-MuLV is a fusion between retroviral *gag* sequences and the c-*abl* gene, resulting in a 160-kDa *gag/abl* fusion protein denoted $P160^{gag/abl}$ or $P160^{v\text{-}abl}$ (Fig. 1). $P160^{gag/abl}$ includes the p15, p12, and a part of p30 sequences of Gag at the N-terminus fused to 1008 amino acids derived entirely from c-Abl. Like the native Gag protein, the Gag-Abl fusion protein has a myristate fatty acid moiety covalently linked through the NH_2 group of the N-terminal glycine [24]. For unknown reasons, these Gag proteins are not efficiently cleaved from the v-Abl fusion protein by the viral protease in cells infected with Ab-MuLV and Mo-MuLV. Another naturally isolated strain of Ab-MuLV was found to carry a smaller form of *gag/abl*, P120. The P120 genome has an internal deletion in the *abl* sequences relative to P160, resulting in a smaller protein. Sequence analysis of the two strains have shown that they have identical recombination breakpoints, suggesting that they both arose from the same primary transduction event, with P120 later derived from the P160 strain.

The $P160^{v\text{-}abl}$ gene, when expressed in fibroblasts or hematopoietic cells without other retroviral sequences, is able to recapitulate the full range of transforming activity demonstrated by Ab-MuLV, and hence the question of transformation by Ab-MuLV may be focused on understanding the function of the v-*abl* gene. Like the v-Src protein, the v-Abl protein has protein-tyrosine kinase activity and is able to autophosphorylate on tyrosine in vitro [25,26]. Naturally occuring deletion mutants of Ab-MuLV defined the need for the tyrosine kinase domain of the protein, encoded by the Abl portion of the molecule, for transformation [27,28]. Temperature-sensitive mutations in kinase function are conditional for transformation, implicating the kinase activity directly in transformation [29,30]. Studies of the localization of the v-*abl* protein by subcellular fractionation have shown that v-Abl is a cytoplasmic protein, with a substantial portion associated with the plasma membrane and the detergent-insoluble matrix [20,31]. Indirect immunofluorescence has confirmed the association of v-Abl with the inner surface of the plasma membrane and with focal adhesion plaques, similar to the

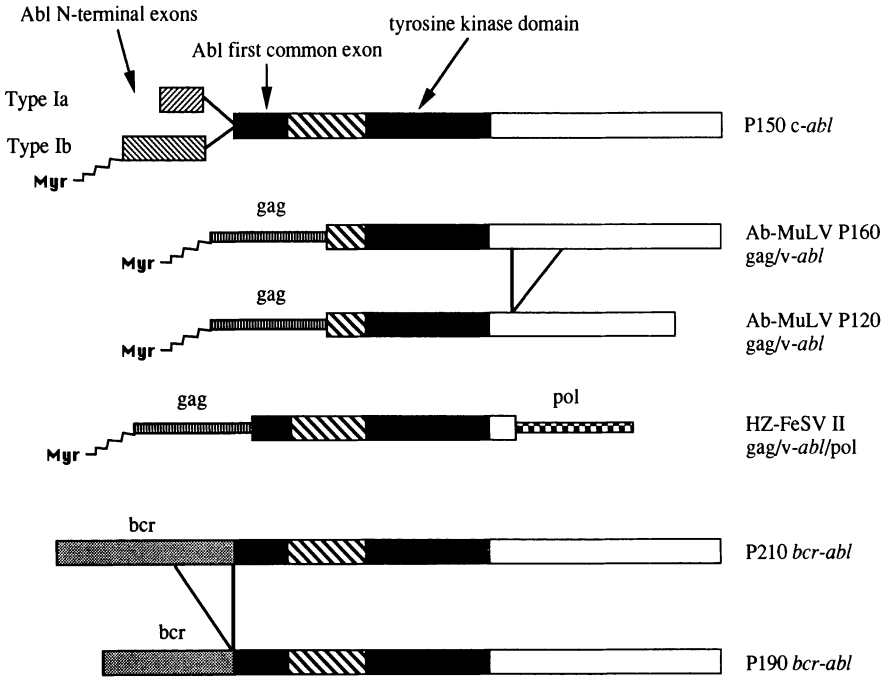

Figure 1. Abelson tyrosine kinase family members. Schematic representation of Abl proteins, with the tyrosine kinase domain shown in black. The deletions of sequences between P160 and P120$^{v\text{-}abl}$, and P210 and P190$^{BCR/ABL}$, are indicated by the lines.

distribution of v-Src [32,33]. Myristoylation of v-Abl and v-Src is thought to be important for membrane association, as myristoylation-defective mutants of v-Src have decreased membrane association [34]. Myristoylation-defective mutants of v-Abl and v-Src are unable to transform fibroblasts [35,36].

The reason for the propensity of Ab-MuLV to induce pre-B lymphoma after intraperitoneal inoculation is not completely understood. In comparative studies of murine retroviruses with different tissue tropisms, the long terminal repeat (LTR) sequences, which contain *cis*-acting regulatory elements for the control of viral transcription, were implicated as controlling disease specificity induced by the virus. However, when the LTR of Ab-MuLV was replaced by the LTR from an erythrotropic retrovirus (Friend MuLV), a fibrotropic retrovirus (Harvey murine sarcoma virus) [37], or a pleiotropic hematopoietic retrovirus (myeloproliferative sarcoma virus or MPSV) [38], the pre-B specificity of Ab-MuLV after intraperitoneal inoculation did not depend on the nature of the LTR, given that a reasonable level of expression of the v-*abl* gene product was obtained. Thus, the v-*abl* gene itself has a certain propensity to transform this particular target cell after intraperitoneal inoculation. At least part of this specificity may depend on the route of infection and the access of the virus to target cells, as direct

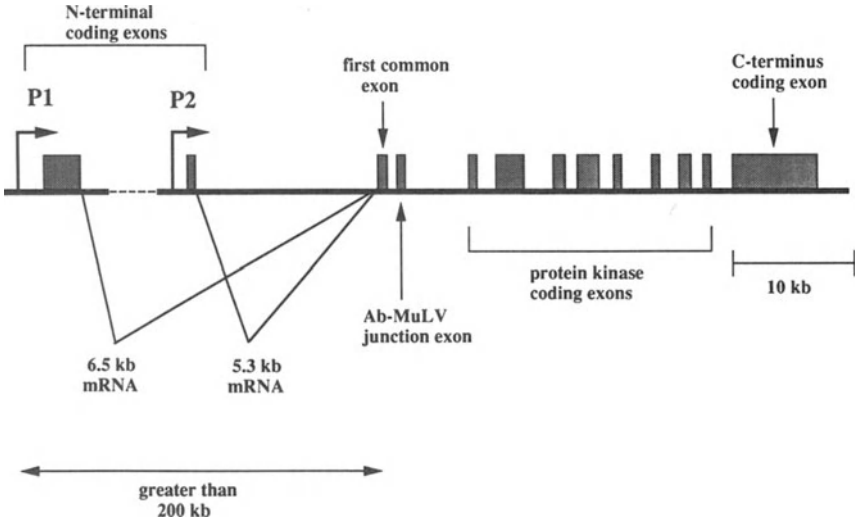

Figure 2. Structure of the mammalian c-*abl* gene. For details, see text.

infection of bone marrow by v-*abl* yields a broader spectrum of malignancy (see below).

An independent retroviral transduction of c-*abl* appears to have occurred in the generation of the Hardy-Zuckerman feline sarcoma virus type II (HZ-FeSV II), which causes fibrosarcoma in cats [39]. The structure of the HZ-FeSV II transforming gene is shown in Fig. 1. The gene is a *gag-abl-pol* fusion, with the FeSV gene containing more 5′ *abl* sequence and lacking much 3′ *abl* sequence compared with $P160^{gag/abl}$ of Ab-MuLV. When expressed in a murine retroviral backbone, the HZ-FeSV II *abl* gene induced a leukemia indistinguishable from that induced by Ab-MuLV [40]; the reciprocal experiment, testing the ability of $P160^{gag/abl}$ to induce feline sarcoma, has not been reported.

The c-*abl* gene: Structure, function, and activation

Utilizing probes from v-*abl*, the c-*abl* gene was cloned and characterized [21,41]. The mammalian c-*abl* locus is fairly complex in organization and expression (Fig. 2). The gene is composed of 13 exons spread over about 250–300 kb of genomic DNA. Two distinct promoters, P1 and P2, independently initiate transcription at either of two distinct first exons, which then splice onto common downstream exons, yielding two major mRNA transcripts of 6.5 and 5.3 kb. The P2 promoter is located about 17 kb from the first common exon, but the P1 promoter is located at least 200 kb upstream from the first common exon, making this one of the largest introns

known [42]. The remainder of the *abl* common exons, including one large 3′ exon, are confined to about 30 kb of genomic DNA. The c-*abl* promoters are typical of genes of a 'housekeeping' nature, lacking TATA sequences and having high GC content. The two RNA transcripts are relatively ubiquitously expressed in hematopoietic and nonhematopoietic adult tissues, and throughout mouse embryonic development [43]. The only variation in this transcription pattern is in haploid cells in the testis, where a 4.2-kd mRNA is also found. This RNA differs from the 5.3-kb message only in its 3′ untranslated sequences; the mechanism of production of this alternate form and its possible functional significance are unknown [44]. Cloning of mammalian c-*abl* cDNAs has revealed two major species with two alternatively spliced 5′ ends, corresponding to the two distinct 5′ exons [45,46]. There are two additional minor cDNA species identified in mouse cells that are not conserved in humans and are thought to be of minor importance.

The two c-*abl* mRNAs code for two major c-Abl proteins, which differ in sequence only at their N-termini. The two forms are denoted c-Abl Ia and Ib in humans, and c-Abl I and IV, respectively, in mice. The c-Abl Ia/I protein is 1122 amino acids in length, while the c-Abl Ib/IV protein is 1142 amino acids long. Both proteins migrate together on SDS-polyacrylamide gels as a single species of about 150 kDa. As mentioned above, the c-Abl proteins are members of a family of nonreceptor protein-tyrosine kinases, of which the Src protein is the prototype, all of which share the ability to phosphorylate cellular proteins on tyrosine; however, Abl is distinct from members of the Src family by the existence of a large 80 kDa domain C-terminal to the kinase region, while the Src family members all terminate just after the kinase region. Thus, formally, *abl* belongs in a separate subfamily from *src* [47]. Another potential member of the *abl* subfamily, denoted *arg* for Abl-related gene (also known as *abl*-2), has been cloned by differential hybridization [48]. Arg also contains the large C-terminal domain characteristic of Abl [49], but whether there are additional members of this subfamily is unknown.

The c-*abl* gene appears to be highly conserved throughout metazoan evolution, but the physiological function of *abl* is unknown. The c-*abl* locus in Drosophila has been studied extensively [50]. The Drosophila c-*abl* gene is highly expressed in neuronal axons of the developing central nervous system. Homozygous null mutations of *abl*, while exhibiting no gross CNS structural abnormalities, have a high degree of pupal lethality with reduced fecundity and rough eyes in surviving adults. The severity of the phenotype can be dramatically enhanced by placing the *abl* null homozygous genotype in other genetic backgrounds, such as flies mutant in fasciclin I or haploinsufficient for certain genes such as the disabled (*dab*) locus [51]. Such mutants exhibit embryonic lethality and severe disruption of the CNS, suggesting that in flies *abl* plays some fundamental role in organization and pathfinding of CNS neurons. In contrast, homozygous inactivation of the c-*abl* gene in mice leads to runted growth and immunodeficiency, mainly from

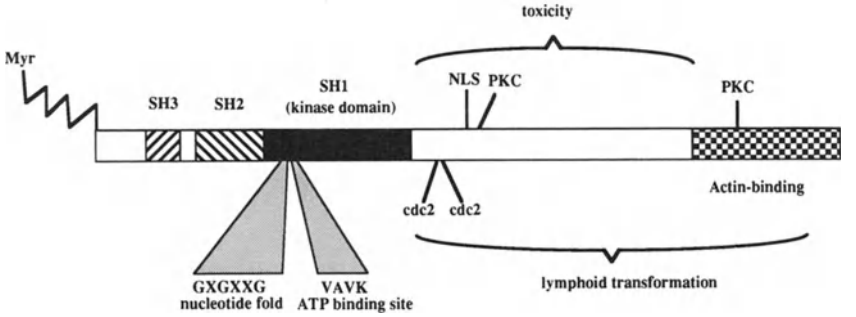

Figure 3. Structural and functional domains of the c-Abl Ib protein. For details, see text. Myr = myristate fatty acid group; NLS = nuclear localization signal; PKC = protein kinase C phosphorylation site; cdc2 = $p34^{cdc2}$ phosphorylation site.

a deficiency in mature lymphocytes, with CNS structure apparently normal [52,53]. This suggests that c-*abl* has a physiologically different role in flies and mammals.

Recently, structural and functional studies of the c-*abl* gene have defined several domains (Fig. 3). Studies of the subcellular localization of the ABL proteins in fibroblasts have shown that the c-Abl Ib/IV protein is largely nuclear in location, with a portion present at the inner surface of the plasma membrane and in adhesion plaques [33]. The subcellular localization of the c-Abl Ia/I protein is unknown. The nuclear localization of the c-Abl Ib/IV protein is dependent on the presence and function of a basic pentalysine (K5) motif in the C-terminus, which is homologous to the nuclear localization signal of SV40 large T antigen [33]. There is a signal for myristoylation in the first 7–14 amino acids of c-Abl Ib [54], and this protein has been shown to be myristoylated at an N-terminal glycine residue [55], while the c-Abl Ia protein is predicted from its sequence not to be myristoylated. The myristate group in c-Abl Ib is necessary for membrane localization of a fraction of the protein, although myristoylation alone is not sufficient to localize all the Ib protein to the membrane. It is expected that the myristoylated form of Abl may have a specific protein receptor in the membrane, like those demonstrated for Lck [56] and Src [57]. The myristate group is also necessary for transforming alleles of Abl to transform fibroblasts, but is not required for transformation of hematolymphoid cells by Abl [35].

In a sequence comparison with Src, three major regions of homology between Src and Abl are identified, denoted the Src homology or SH regions [58,59]. The first Src homology region, SH1, defines the tyrosine kinase domain, which studies of deletion, conditional, and kinase-inactive mutants of Abl have demonstrated is required for transformation. Like other protein kinases, the Abl kinase domain contains canonical sequences for the binding of the adenine nucleotide of ATP (GXGXXG), with a

conserved downstream VA(V/I)K motif for hydrogen bonding of the β phosphate of ATP. Expression of the Abl kinase domain in *E. coli* or by in vitro translation confirms that this minimal region is able to function as a tyrosine kinase [60]. Immediately N-terminal of the kinase domain in Src and Abl is a second region of homology of about 100 amino acids, the SH2 domain. Although not strictly required for catalytic activity, mutations in SH2 generally decrease the transforming activity of oncogenic tyrosine kinases [61] and can confer host-range-dependent transforming activity [62]. Recently, the SH2 domains of Abl and Fps have been shown to bind to tyrosine-phosphorylated cellular proteins with high affinity [63,64], perhaps mediating the formation of protein–protein complexes between protein-tyrosine kinases and substrates, which may be important for signal transduction [65] (see below). The final Src homology region, SH3, while present in both Abl and Src, is missing from some other Src family members such as Fps. In Src and Abl, the SH3 domain is about 50 amino acids in length, located just N-terminal to SH2. SH3 appears to be a negative regulatory domain of Abl, as some mutations in this region serve to activate the transforming ability of c-Abl [55], (see below).

The large C-terminal domain of Abl contains several known functions. Besides the nuclear localization signal mentioned earlier, there are two sites for phosphorylation of Abl by protein kinase C [66]. In addition, c-Abl appears to be differentially phosphorylated during the cell cycle by the mitotic kinase $p34^{cdc-2}$ at several sites, all in the C-terminal domain; only two of these sites have been precisely identified [67]. The functional implication of phosphorylation of Abl by these kinases is unknown. At the extreme C-terminus of Abl is a domain that mediates the association of Abl with the F-actin cytoskeleton, a finding of unknown significance [33,68]. Two other functions of the Abl C-terminus, a requirement for lymphoid cell transformation and a phenomenon known as toxicity, are as yet poorly defined genetically. There appears to be a relative requirement for the C-terminus of v-Abl for transformation of lymphoid cells, as v-Abl mutants truncated at the C-terminus, while unaffected in their ability to transform 3T3 cells, exhibit a greatly reduced frequency of lymphoid transformation in vitro and in vivo [69,70]. The precise sequences responsible for reduced lymphoid transformation have not been mapped. The phenomenon of toxicity of Abl proteins, observed only in fibroblast cell types, is manifest in a number of different ways and is likewise poorly understood. With transforming alleles of *abl*, infection of Balb/c 3T3 fibroblasts leads to a transient transformed phenotype, followed by cell death [71]. Cotransfection of NIH 3T3 cells by transforming or nontransforming alleles of *abl* with a selectable marker gene shows that cells expressing both genes are recovered at a much lower frequency than control cells transfected with the marker gene alone [72]. With nontransforming alleles of *abl*, it is possible to derive subclones of transfected cells that express c-Abl at levels 10- to 20-fold increased over the level of endogenous Abl. Such clones grow much more slower than

parental NIH 3T3 cells by a factor of 2.5–3.0, and there is a strong tendency with simple passage of these clones in culture to select for variants that lose Abl expression, even when grown under continued selective pressure. These 'revertant' lines again grow at normal rates [73]. Thus there is some negative effect associated with Abl expression at the cellular level. The toxic effect of *abl* requires an active kinase domain and an intact C-terminus linked in *cis* in the transfected *abl* gene, but the precise sequences in the C-terminus required for toxicity have not been defined. A truncation mutant of $P160^{v\text{-}abl}$, denoted P130, which terminates around amino acid 850, is defective for lymphoid transformation but is still toxic for fibroblasts, indicating that the two functions can be genetically separated [70].

Overexpression of either c-Abl Ia or Ib via a retroviral expression vector is insufficient for transformation of fibroblasts or hematolymphoid cells [33,55], and, as mentioned above, overexpression of c-Abl Ib instead has a profound negative effect on fibroblast cell growth. Therefore, in contrast to protooncogenes like c-*myc*, where simple overexpression of the normal gene product is sufficient for transformation, *abl* belongs to a class of proto-oncogenes that must be altered by specific mutation in order to transform cells. There are three mechanisms currently known by which the transforming ability of c-*abl* may be activated. The largest known class of activated *abl* genes contain mutations in the N-terminal regulatory region SH3, including both deletions and small insertions [55,74]. Point mutations can also contribute to the oncogenicity of *abl*. Comparison of the transforming activity of a series of recombinants between v- and c-*abl* led to the discovery that some isolates of the P160 strain of v-*abl* carry a point mutation in the tyrosine kinase domain that changes the phenylalanine at position 420 to valine (F420V), a mutation that confers weak transforming ability on c-*abl* in the absence of other mutations [75]. As another example, a point mutation in the C-terminus of *abl* contributes to the transforming potency of a *gag/abl* fusion construct [76]. Finally, addition of certain exogenous sequences to the N-terminus of Abl in place of Abl first exon sequences can activate *abl* independent of the first two mechanisms [77]. One example of this mechanism is retroviral *gag* sequences, which have been shown to dominantly activate the transforming ability of *abl* independent of SH3 mutation. Interestingly, the $P160^{v\text{-}abl}$ gene has been activated by all three mechanisms, since the addition of Gag sequences to the Abl N-terminus has also resulted in partial deletion of the SH3 domain. Activation of c-Abl Ib by any of these mechanisms is associated with a change in the localization of the protein from the nucleus to the cytoplasm and plasma membrane [33]. In the c-Src protein, phosphorylation of the tyrosine at position 527 has a negative regulatory effect on kinase activity and transforming ability, and mutation of this residue to phenylalanine or deletion (as occurs in v-Src) weakly activates transforming ability [78]. Abl lacks a homolog of tyrosine 527, so that this mechanism of control of Src transforming activity has no counterpart in Abl.

Fibroblasts transformed by activated forms of *abl* display all the usual correlates of the transformed state, including morphological changes, loss of 'contact inhibition.' decreased adherence, changes in ionic permeability and glucose transport, growth in semisolid medium and low serum, and disruption of the cytoskeleton. They also contain greatly increased levels of phosphotyrosine-containing proteins and elevated levels of phosphotidylinositol breakdown products. One of the most prominent phosphotyrosinated species in *abl*-transformed cells is the Abl protein itself [25], which contains phosphotyrosine at two major sites, one of which maps within the kinase domain at position Y412 [79,80]. While autophosphorylation of v-Src or v-Fps is thought to increase the catalytic activity of the kinase, the effect of autophosphorylation on Abl function or kinase activity is unclear. While c-Abl is not detectably phosphorylated on tyrosine in nontransformed cells [81] or after in vitro translation [82], in both cases tyrosine kinase activity may be demonstrated in vitro after immunoprecipitation of c-Abl and washing of the immune complex at high stringency [83]. In addition, phosphotyrosine can be detected on c-Abl proteins after overexpression 100- to 500-fold in Cos cells or Sf9 insect cells [82]. Taken together, these observations suggest that c-Abl tyrosine kinase activity is reversibly inhibited in vivo by a cellular factor that is removed by immunoprecipitation or is titrated out by vast overexpression of c-Abl. An attractive hypothesis is that this putative inhibitor may interact with the negative regulatory SH3 domain, such that mutations in SH3 domain lead to loss of inhibition of the *abl* kinase by the inhibitor, allowing expression of the latent kinase activity of Abl. Thus, activating mutations in c-*abl* unmask the inherent kinase activity of the protein in vivo, leading to autophosphorylation, increased levels of phosphotyrosine-containing proteins in the cell, and transformation. It is not clear whether activating mutations of c-*abl* directly affect the intrinsic kinase activity of the Abl protein or whether they activate c-Abl by affecting some other parameter, such as interactions with inhibitors, alterations in substrate specificity, or changes in subcellular localization. Although some workers have found an increase in the intrinsic kinase activity of transforming alleles of *abl* as compared with c-*abl* [84], others have not [80,85].

The molecular mechanism of transformation of cells by the Abl tyrosine kinase, or indeed by any of the tyrosine kinase oncogenes, is unknown. Since transformed cells in culture have reduced growth factor requirements, they might secrete their own growth factors, as proposed in the autocrine hypothesis of Sporn and Todaro [86]. Fibroblasts transformed by v-*abl* produce a number of transforming growth factors, including transforming growth factor α (TGF-α) [87], which has homology to epidermal growth factor and utilizes the EGF receptor. A mutant Swiss 3T3 fibroblast cell line, lacking the EGF/TGF-α receptor, is morphologically transformed by v-*abl* but is nontumorigenic in nude mice, in contrast to the parental cells [88]. Thus, autocrine stimulation mediated by TGF-α may be required for the full

tumorigenic phenotype induced by v-*abl* in fibroblasts. Because the tyrosine kinase activity of Abl is required for transformation, it is reasonable to expect that tyrosine phosphorylation of one or more particular substrate proteins is crucial for transformation by Abl. Despite extensive searches for substrates of protein-tyrosine kinases, no substrates necessary for transformation or for normal function have been identified [36,89], nor is it even known if substrates phosphorylated by transforming kinases are hyperphosphorylated normal substrates or aberrant substrates. Recently, a number of molecules involved in growth-factor signal transduction and cell growth have been found to be substrates of receptor tyrosine kinases, to form stable complexes with ligand-activated growth-factor receptors, and in some cases to have their functional properties altered by tyrosine phosphorylation. This list of substrate proteins includes phospholipase C-γ, the 85-kDa subunit of the phosphatidylinositol 3-kinase (PI 3-kinase), c-*raf*, and *ras*-GAP [for review, see 65]. SH2 domains have been identified in some of these proteins and may mediate the formation of protein–protein complexes with activated growth-factor receptors by binding to autophosphorylated receptors. Abl has been shown to be associated with some of these molecules, notably the PI 3-kinase [90] and a 62-kDa GAP-associated protein [63,91]. Although none of these substrates has yet been shown to be critical for transformation, this remains an important and active area of oncogene research.

Abl in human malignancies: CML and the Philadelphia chromosome

Chronic myelogenous leukemia (CML) is a hematologic malignancy characterized by increased numbers of maturing myeloid cells and their progenitors in peripheral blood, bone marrow, liver, and spleen [for review, see 92]. CML has a triphasic clinical course and serves as a paradigm of multistep carcinogenesis. The early so-called chronic phase of the disease is marked by greatly increased numbers of maturing myeloid cells. Terminal differentiation of cells to the postmitotic state is maintained, resulting in a large elevation in the peripheral blood granulocyte count. Although there are subtle abnormalities of granulocyte and platelet function, these rarely lead to significant complications. Thus, the chronic phase differs from a de novo acute leukemia, which is characterized by a profound block in differentiation and overgrowth of blood and bone marrow with immature, proliferating cells. The chronic phase of CML may be managed by several different treatments, and patients usually have a nearly normal quality of life. However, the chronic phase is inherently unstable, and after a variable period of time ranging from weeks to several years (mean interval 3–4 years), the disease enters a period of acceleration, where the capacity of the malignant cells to terminally differentiate becomes progressively impaired, resulting in the appearance of more immature myeloid cells in the peripheral blood, often accompanied by basophilia and thrombocytosis. Shortly there-

after, the terminal (and inevitably fatal) blast crisis stage appears, characterized by proliferating immature blast cells. The phenotype of the blast cells may be myeloid (65% of cases), B-lymphoid (30% of cases), or megakaryocytic, erythroid, T-lymphoid, or undifferentiated (<5% of cases). Myeloid blast crisis cells are morphologically similar to myeloblasts or promyelocytes, and express myeloid-specific cell surface antigens and cytoplasmic enzymes. Lymphoid blast crisis cells are phenotypically pre-B leukemia cells, express terminal transferase and common acute lymphoblastic leukemia antigen (CALLA, CD10), and have immunoglobulin heavy-chain gene rearrangements and express cytoplasmic μ chains. Clinically, CML is usually grouped with polycythemia vera, essential thrombocythemia, and myeloid metaplasia as one of the myeloproliferative diseases.

CML was the first human cancer to be associated with a consistent chromosomal abnormality. Working in Philadelphia, Nowell and Hungerford in 1960 described a small chromosome 22 (now called the Philadelphia chromosome or Ph^1) in metaphase preparations from patients with CML [93]. In 1973, Rowley demonstrated that Ph^1 typically results from a reciprocal or balanced translocation between the long arms of chromosomes 9 and 22: t(9;22)(q34.1;q11.21) [94]. Thirty years later, the Philadelphia chromosome remains the best tumor-specific marker for any human malignancy. Using the Philadelphia chromosome as a marker, early cytogenetic studies established that Ph^1 was present in granulocyte, monocyte, and erythroid precursors and in megakaryocytes [for example, see 95]. The Ph^1 marker is not found in bone marrow fibroblasts (or other mesenchymal tissues), even in cases of myelofibrosis (which is frequently associated with CML) [96]. In the lymphoid compartment, B lymphocytes usually carry the Ph^1 marker, but peripheral blood T cells usually do not. These results suggest that CML is a clonal disorder arising in a multipotential hematopoietic stem cell. The lack of consistent involvement of the T-lymphoid lineage may be a consequence of preexisting long-lived normal T cells that predominate during the chronic phase, and it is possible that small numbers of Ph^1-positive T cells are present in CML patients but cannot be detected. When blast crisis develops, the blast cells are invariably found to carry the Ph^1 marker, confirming that the blast crisis cells arise from the same leukemic clone as the chronic phase. Although rare, several cases of Ph^1-positive T-cell blast crisis have been reported [97], suggesting that the disease involves a pluripotent cell capable of differentiating to T-lymphoid cells as well as along B-lymphoid and myeloid lines. Taken together, these studies strongly support the concept that CML is a malignancy involving the pluripotent hematopoietic stem cell.

The elucidation of the structure of the Philadelphia chromosome is a triumph of modern molecular biology [for recent reviews, see [98–100]. Early studies of rodent–human somatic cell hybrids containing Ph^1 demonstrated that the c-*abl* protooncogene, normally located on the long arm of chromosome 9 (band q34), is translocated to the Ph^1 chromosome in CML [101]. Fine structure mapping of the c-*ABL* locus in CML cells established

that the breakpoints occurred 5′ to the body of the *ABL* gene, and in one patient occurred within 14.5 kb of *ABL* sequences [102]. However, even though as much as 40 kb of human genomic DNA that mapped 5′ to the c-*ABL* sequences were cloned, probes derived from that genomic DNA failed to detect additional breakpoints in samples from other CML patients. This mystery was resolved when it was found that probes derived from the chromosome 22 side of the Ph^1 breakpoint detected rearrangements in virtually all CML patients [103]. When the various breakpoints were molecularly cloned, it became clear that the breakpoints on chromosome 9 occurred at variable and sometimes great distances (hundreds of kilobases) upstream of the c-*ABL* locus, explaining why few rearrangements can be detected using *ABL* probes, which contain only the 3′ end of the locus. In contrast to the variability of breakpoints on chromosome 9, the breakpoints on chromosome 22 all fell in a restricted 5.8-kb area, which was termed the *breakpoint cluster region* or *bcr*.

The *BCR* gene is composed of 20 exons spanning about 70 kb, with the 5.8-kb breakpoint cluster region located in the center of the gene and, like the c-*ABL* gene, is oriented with the 5′ end towards the centromere. The *BCR* gene expresses 4.5- and 6.7-kb mRNA transcripts, which, like those of the c-*ABL* gene, appear to be ubiquitous, and are detected in all hematopoietic cells as well as fibroblasts and cells of epithelial origin [104]. CML cells express two novel products: an altered *ABL*-related mRNA of about 8.5 kb [104] and an altered form of the c-*ABL* protein with a molecular weight of 210 kDa [105]. Molecular cloning of the cDNA corresponding to the altered 8.5-kb transcript established that this was a fused transcript consisting of 5′ *bcr* sequences joined to 3′ c-*ABL* sequences [106]. Antibodies raised against synthetic peptides predicted by the *BCR* and *ABL* sequences immunoprecipitated the altered *ABL* protein of CML cells and confirmed that it was a hybrid protein representing the fusion of Bcr and Abl polypeptides predicted from the sequence of the cDNA [107].

In summary, the Philadelphia chromosome results in a fusion of the genes for *BCR* and c-*ABL*, with the breakpoint in the *BCR* gene occurring in a restricted area called the breakpoint cluster region and the breakpoint in the c-*ABL* gene occurring somewhere in a very large first intron. Following transcription, splicing, and translation, a 210-kDa *BCR/ABL* fusion protein (denoted $P210^{BCR/ABL}$) is produced. Although the reciprocal product to the Ph^1 translocation also results in the fusion of the 5′ end of the c-*ABL* locus with the 3′ end of the *BCR* gene, the product of this chimeric gene has not been consistently identified in CML cells. In addition, the protooncogene c-*SIS*, which encodes platelet-derived growth factor, is also translocated from chromosome 22 to chromosome 9 in CML, and has been postulated to play a role in the myelofibrosis that often accompanies CML. However, the c-*SIS* gene is located a substantial distance away from the breakpoint, and there is no evidence that it is involved in any way in the pathogenesis of CML.

The Ph^1 chromosome is also found in approximately 30% of cases of

adult acute lymphoblastic leukemia (ALL), 10% of pediatric ALL, and about 1% of adult acute myelogenous leukemia (AML). Some patients with Ph^1-positive ALL have recently been shown to have a distinct Abl-related protein of molecular weight 185–190 kDa [108]. Molecular cloning and sequencing of cDNAs from these patients have revealed a novel form of the *BCR/ABL* translocation, with the breakpoint on chromosome 22 in the first intron of the *BCR* gene instead of the usual 5.8-kb breakpoint cluster region [109]. This leads to expression of a 190-kDa fusion protein consisting of sequences from the first exon of the *BCR* gene joined to the second *ABL* exon, differing from P210, which contains sequences from the first 11 or 12 exons of *bcr* in the hybrid protein. Because the P190 form of *BCR/ABL* has been found in cases of Ph^1-positive AML, it appears that the P190 protein is not specifically linked to lymphoid leukemia [110]. However, it may be that the P190 form of *BCR/ABL* is associated only with cases of de novo acute leukemia, since P190 is rarely if ever seen in the chronic phase of CML [111]. While all documented cases of CML have traditional breakpoints in the 5.8-kb *bcr* region, giving rise to the P210 form of *BCR/ABL*, some patients who present with Ph^1-positive ALL and AML also have traditional *bcr* breakpoints and express P210. As a rule, these patients have persistence of the Ph^1 chromosome in remission, suggesting that they represented cases of CML presenting in blast crisis after an unrecognized chronic phase. In contrast, most ALL patients with the more 5′ breakpoint become Ph^1-negative during remissions and do not exhibit the additional cytogenetic abnormalities typical of CML blast crisis, suggesting that they represent transformation of a cell type that is more restricted in its differentiation potential than a pluripotent stem cell [112]. However, patients who appear to violate these rules have been described [113,114]. A central unanswered question is whether the difference in disease spectrum associated with the two forms of *BCR/ABL* is due to some intrinsic difference in the properties of the proteins themselves. An alternate but not mutually exclusive explanation is that different translocation breakpoints on chromosome 22 are favored in different hematopoietic cell types, with the traditional *bcr* breakpoint favored in stem cells and the breakpoint in the *BCR* first intron favored in committed lymphoid (or myeloid) progenitor cells. The recent development of animal model systems of the Philadelphia-positive leukemias may allow an answer to this important question.

Properties of *BCR* and *BCR/ABL*

The substitution of Bcr sequences for the N-terminal exon of c-Abl results in a large increase in the tyrosine kinase activity of the fusion protein [105]. Thus, the Bcr/Abl proteins are examples of Abl proteins that are activated by the third mechanism listed above. The SH3 domain of Abl is encoded starting with *ABL* exon 2 (the first common exon) and is present intact in

the Bcr/Abl proteins. In addition, direct sequencing of the *ABL* portion of the $P210^{BCR/ABL}$ gene from the CML cell line K562 reveals no changes in nucleotide sequence between human c-*ABL* and P210, suggesting that point mutations like those present in v-*abl* have not occurred. The specific mechanism by which *BCR* activates *ABL* is unknown. The normal product of the *BCR* gene is a 160-kDa cytoplasmic phosphoprotein of unknown function [115], is rather ubiquitously expressed in both hematopoietic and non-hematopoietic tissues [104], and appears to have a closely associated serine/threonine kinase activity. The serine/threonine kinase activity is found associated with Bcr even after in vitro translation of the *BCR* gene in reticulocyte lysates [116], but is unlikely to be the *BCR* gene product itself, as the primary sequence of Bcr bears no homology to any of the known serine/threonine kinases [117]. There is, however, a conserved ATP-binding motif encoded by the first exon of *BCR*. There is a recent report that Bcr has GTPase activating (GAP) activity for $p21^{rac}$, a *ras*-related G protein [118], but the region of Bcr with GAP activity is in the C-terminal region of the protein, which is not present in the Bcr/Abl proteins, so that the relevance of this finding to transformation or leukemogenesis by Bcr/Abl is unclear. Deletion mapping of the Bcr sequences in Bcr/Abl, using endogenous phosphotyrosine content [85] or Rat-1 fibroblast transformation [119] as assays for activation, suggests that the first exon of Bcr (found in P190) alone contains all the information necessary to activate Abl and also appears to increase the association of Bcr/Abl proteins with the detergent-insoluble matrix and F-actin cytoskeleton [85]. This Bcr first exon may specifically interact with the N-terminal SH2 domain of Abl, perhaps inducing a conformational change in the Bcr/Abl fusion protein that deregulates the tyrosine kinase activity [120].

Like v-Abl, $P210^{BCR/ABL}$ has the ability to transform established factor-dependent myeloid [121] and lymphoid [122] cell lines to growth factor independence by a mechanism that probably does not involve autocrine growth factor production. In some cases, the mechanism of transformation appears to be the constituitive activation of cellular proteins normally involved in growth-factor signal transduction [123]. Unlike v-Abl, however, the P210 protein is unable to transform NIH 3T3 fibroblasts [124], probably because it lacks a myristate group [35], although it is able to partially transform another fibroblast cell line, Rat-1 [125]. P210-transformed Rat-1 cells form small colonies in soft agar and noninvasive tumors in nude mice. v-Myc is able to cooperate with P210 in this assay to induce large colonies in agar and invasive tumors in nude mice [125]. When the P210 protein is expressed (by retroviral gene transfer) in long-term bone marrow culture under conditions that favor lymphoid (Whitlock-Witte) or myeloid (Dexter) cell growth, there is a clonal or oligoclonal outgrowth of immature B-lymphoid cells, which remain dependent of the feeder layer and are not fully tumorigenic [126,127]. Some clones expressing P210 progress to tumorigenicity upon continuous in vitro culture, suggesting that additional

mutational events must occur to produce the fully transformed phenotype. The target cell for P210 in these experiments appears to be similar to the Ab-MuLV target cell.

P190 has greater transforming activity in these assay systems than P210. P190 is more efficient than P210 in transformation of Rat-1 cells [84], and P190-infected Whitlock-Witte cultures grow more quickly and reach greater density than cultures infected with matched stocks of P210 virus [128]. Thus, P190 appears to be a more 'potent' oncogene in some way than P210, and it is tempting to postulate that this may in part explain the epidemiological association of P190 exclusively with the more aggressive acute leukemias in humans. The hallmark of CML is the overproduction of myeloid cells without a block in the ability of these cells to differentiate, and it is reasonable that P210, as a weak oncogene, might induce this phenotype upon expression in a pluripotent cell type. In support of this, a modification of the standard Whitlock-Witte culture conditions allows the outgrowth of clonal lines after P210 infection that have characteristics of early B-lymphoid progenitors. These cells are growth stimulated by P210 but maintain full differentiative capacity and are able to reconstitute the B-lymphoid compartment of scid mice [129]. These experiments confirm the ability of P210 to stimulate the growth of hematopoietic cells without blocking differentiation. Similar experiments with P190 have not been reported, but one might predict that the transforming ability of P190 might be too 'strong' to allow preservation of differentiation of this nature.

Expression of *abl* and *BCR/ABL* in animal models

A great deal has been learned about the biochemistry and genetics of transformation by activated alleles of *abl* by expressing these genes in various tissue culture systems, but full understanding of their leukemogenic properties can only come from studying their expression within the hematopoietic system of a living animal. Two distinct strategies have been employed: the creation of transgenic strains of mice that carry activated *abl* genes or the transduction of activated *abl* genes into murine bone marrow cells using retroviral vectors and bone marrow transplantation.

Transgenic mice have been generated that carry the v-*abl* gene under the control of the SV40 early region promoter and the immunoglobulin heavy chain gene enhancer (Eμ). These mice develop plasmacytomas but not pre-B lymphoma [130]. This suggests that the Eμ-directed transgene may not be expressed at an early enough stage to transform the traditional Abelson target cell for the development of pre-B lymphoma, and B-cell malignancy subsequently develops at a more differentiated step in development. The plasmacytomas are clonal with respect to IgH or κ gene rearrangements, secrete IgA or IgG, and have rearrangements of the c-*myc* locus. In addition, the progeny of a cross between Eμ-v-*abl* transgenic mice and a strain

bearing a Eμ-c-*myc* transgene resulted in the rapid development of oligoclonal plasmacytomas, confirming the cooperation between these two oncogenes. Transgene expression was detected in hematopoietic tissues of the Eμ-v-*abl* mice before the development of overt plasmacytoma, but the mice were hematologically normal during this period.

Transgenic mice carrying the $P190^{BCR/ABL}$ gene have also been generated by placing the P190 gene under the control of the mouse metallothionein promoter [131]. The P190 transgenic animals rapidly developed fatal acute leukemias shortly after birth, or in some cases are moribund at birth with leukemia that may have developed in utero. These leukemias were of lymphoid type in most cases, with a few cases of acute myeloid leukemia, and were of such rapid onset that the affected animals could not be bred. Interestingly, although transgene expression was detected in a variety of tissues, including muscle, brain, and spleen, without induction with heavy metals, only malignancies of the hematopoietic system were observed. These experiments strongly implicate the P190 protein in the pathogenesis of acute leukemia, but none of the animals exhibited the prominent granulocytosis characteristic of CML.

Efforts to generate transgenic mice bearing the $P210^{BCR/ABL}$ gene have been somewhat less successful. Several transgenic strains of mice have been generated that carry a facsimile of the human $P210^{BCR/ABL}$ gene under the control of either the Eμ enhancer or the relatively tissue-nonspecific LTR of the myeloproliferative sarcoma virus (MPSV LTR) [132]. Both transgenic strains show a predisposition to develop clonal lymphoid tumors, principally of the T-cell type, but no tumors of myeloid origin have been observed. There appears to be a problem with expression of P210 in transgenic mice, even when under the control of restricted promoters. In these experiments there were smaller numbers of transgenic pups born than would have been expected given historical controls. This decreased yield of transgenic animals was more striking for the MPSV LTR-driven transgene (which is more widely transcribed) than for the Eμ-driven transgene. In both cases, the transgene is silent in the transgenic animals that are born, even in hematopoietic cells, and hematopoiesis appears normal until rare individuals develop T or B lymphomas. These tumors are clonal by analysis of T-cell receptor or IgH gene rearrangements, and the tumor cells express the transgene. Taken together, these observations suggest that there may be some toxic effect of *BCR/ABL* transgenes on embryonic development, and that the transgene must be silenced, perhaps by somatic mutation, for development to proceed to term. Reactivation of the transgene in hematopoietic cells, a rare event, would account for the low frequency of clonal malignancies observed. Other groups have seen no transgenic animals born after repeated attempts to generate mice with a *BCR/ABL* gene under control of the *BCR* promoter, which is quite ubiquitously expressed [131,133]. This 'toxicity' problem seems limited to $P210^{BCR/ABL}$, as v-*abl* and $P190^{BCR/ABL}$ transgenics have been generated without these difficulties [130,131]. In summary, the existing

transgenic model systems provide firm evidence of a causal link between *bcr/abl* and leukemia, but they do not recapitulate the complex biology of CML, where the leukemic cells maintain their ability to terminally differentiate in the chronic phase.

The alternative approach for establishing an animal model for CML is introduction of the *BCR/ABL* gene into hematopoietic stem cells of mice using retroviral gene transfer. Infection of murine bone marrow with a replication-defective, helper virus-free retrovirus carrying the $P210^{BCR/ABL}$ gene followed by bone marrow transplantation into lethally irradiated recipients results in a spectrum of hematopoietic malignancies. In one series of experiments, malignancies of B- and T-lymphoid, erythroid, mast cell, and monocyte-macrophage cell types were observed, but no granulocytosis suggestive of CML was observed [134]. Using a slightly different vector system and different mouse strain, a myeloproliferative syndrome with a striking resemblance to the chronic phase of human CML was observed in transplant recipients, in addition to malignancies of the other cell types [135]. It is not yet clear whether the difference between the two experimental systems involves the vector system, mouse strain, or other factors. Mice with the CML-like syndrome (murine CML) develop very high peripheral blood white cell counts, composed mainly of maturing granulocytes, and hepatosplenomegaly. The *BCR/ABL* provirus can be detected in DNA from spleen, bone marrow, and peripheral blood granulocytes, and early myeloid cells from these sources express the P210 protein, as determined by immunofluorescence, immunohistochemistry, and immunoblotting. The target cell for infection that gives rise to murine CML is an early multipotential hematopoietic progenitor cell, which can give rise to the day 12 spleen colony-forming unit ($CFU\text{-}S_{12}$), and in one case has been shown to be the pluripotent hematopoietic stem cell itself. The disease is transplantable by transfer of bone marrow to syngeneic recipient animals, and clonally related acute leukemias of lymphoid and myeloid origin have been observed in secondary transplant recipients, showing that the leukemic clone can evolve to acute leukemia in a way that mimics the tendency of the human disease to progress to blast crisis [136]. Thus, murine CML resembles the human disease in several fundamental aspects.

One of the questions that can be addressed utilizing this type of bone marrow infection system to express *BCR/ABL* is to determine whether different forms of activated Abl proteins cause different disease phenotypes. In particular, do v-*abl* and $P190^{BCR/ABL}$, which are predominantly associated with pre-B leukemia in mice and humans, respectively, also have the capacity to induce a myeloproliferative syndrome resembling chronic phase CML? Rosenberg, Witte, and coworkers introduced both P210 and $P160^{v\text{-}abl}$ into murine bone marrow by infection with retroviruses that included replication-competent Moloney murine leukemia virus as a helper virus [137]. Some animals infected with P210 developed a granulocytosis suggestive of CML, while animals infected with v-*abl* developed primarily tumors of

monocytic cells. In a similar experiment, Scott and coworkers found that recipients of v-*abl*-infected marrow did exhibit a moderate granulocytosis early after transplant, but that these cells lacked the proviral marker and were not part of the malignant clone [138]. These experiments are complicated by the presence of helper virus, which caused each individual animal to develop multiple diseases and made secondary transplant analysis impossible. Helper-free infection of fetal liver cells with v-*abl* followed by transplantation has also been reported [139]. These animals appear to develop mono- or oligoclonal leukemias, and tumors of monocyte-macrophage cell types, but the occurrence of granulocytosis was not reported. Taken together, the clinical and histopathological picture of disease induced by v-*abl* after bone marrow infection differs considerably from that seen with $P210^{BCR/ABL}$, and the two proteins appear to induce distinct diseases in these animal model systems.

Expression of the P190 gene in the bone marrow infection system in the presence of helper virus gives rise to the same spectrum of hematological malignancies seen with the P210 gene, but with a shorter latency period, again suggesting that P190 is more potent than P210 in leukemogenesis [140]. When P190 is expressed in murine bone marrow by a helper-free retroviral vector, it very rapidly and efficiently induces pre-B lymphoma and tumors of monocyte/macrophage origin. The target cells for the induction of these diseases are lineage-restricted and probably represent committed progenitors. A small number of animals appear to have had stem cell infection by P190 and exhibit a transient myeloproliferative syndrome, followed by rapid evolution of the infected clone to acute leukemia, mimicking blast crisis [141]. These results suggest that P190 may be able to induce CML when expressed in a stem cell, but rapid progression to acute leukemia may preclude identification of human patients with chronic phase CML who express P190.

Summary and future prospects

Although *abl* is one of the oldest oncogenes known, research on this family continues at a brisk pace. In the coming years, we may expect the definition of more functional domains in the c-Abl proteins and progress in understanding the puzzling relationship between transformation and subcellular localization. Identification of proteins interacting with Abl in the cell will be a fruitful area of investigation. The availability of mice strains carrying germline inactivations of the c-*abl* locus should provide insight into the physiological role of *abl* in lymphoid development. Finally, the recent development of animal model systems of the *BCR/ABL* leukemias should accelerate our understanding of the molecular pathophysiology of these diseases, and provide a means of testing new anti-leukemic therapies.

References

1. Rosenberg N, Witte ON: The viral and cellular forms of the abelson (*abl*) oncogene. In: Advances in Virus Research. Academic Press, 1988, pp 39–81.
2. Abelson HT, Rabstein LS: Lymphosarcoma: Virus-induced thymic-independent disease in mice. Cancer Res 30:2213–2222, 1970.
3. Scher CD, Siegler R: Direct transformation of 3T3 cells by Abelson murine leukemia virus. Nature 253:729–731, 1975.
4. Green PL, Kaehler DA, Risser R: Cell transformation and tumor induction by Abelson murine leukemia virus in the absence of helper virus. Proc Natl Acad Sci USA 84:5932–5936, 1987.
5. Risser R, Potter M, Rowe WP: Abelson virus-induced lymphomagenesis in mice. J Exp Med 148:714–726, 1978.
6. Tidmarsh GF, Heimfeld S, Whitlock CA, Weissman IL, Muller-Sieburg CE: Identification of a novel bone marrow-derived B-cell progenitor population that coexpresses B220 and Thy-1 and is highly enriched for abelson leukemia virus targets. Mol Cell Biol 9:2665–2671, 1989.
7. Alt F, Rosenberg N, Lewis S, Thomas E, Baltimore D: Organization and reorganization of immunoglobulin genes in A-MuLV-transformed cells: Rearrangement of heavy but not light chain genes. Cell 27:381–390, 1981.
8. Rosenberg N, Baltimore D, Scher CD: In vitro transformation of lymphoid cells by abelson murine leukemia virus. Proc Natl Acad Sci USA 72:1932–1936, 1975.
9. Potter M, Sklar MD, Rowe WP: Rapid viral induction of plasmacytomas in pristane-primed BALB/c mice. Science 182:592–594.
10. Raschke WC, Baird S, Ralph P, Nakoinz I: Functional macrophage cell lines transformed by abelson leukemia virus. Cell 15:261–267, 1973.
11. Pierce JH, Di Fiore PP, Aaronson SA, Potter M, Pumphrey J, Scott A, Ihle JN: Neoplastic transformation of mast cells by abelson-MuLV: Abrogation of IL-3 dependence by a nonautocrine mechanism. Cell 41:685–693, 1985.
12. Cook WD: Rapid thymomas induced by Abelson murine leukemia virus. Proc Natl Acad Sci USA 79:2917–2921, 1982.
13. Waneck GL, Rosenberg N: Abelson leukemia virus induces lymphoid and erythroid colonies in infected fetal cell cultures. Cell 26:79–89, 1981.
14. Chung S-W, Ruscetti S, Wong PMC: Formation of factor-independent hematopoietic multilineage colonies after abelson virus infection. Blood 71:973–977, 1988.
15. Cook WD, De St. Groth BF, Miller JFAP, MacDonald HR, Gabathuler R: Abelson virus transformation of an interleukin 2-dependent antigen-specific T-cell line. Mol Cell Biol 7:2631–2635, 1987.
16. Mathey-Prevot B, Nabel G, Palacios R, Baltimore D: Abelson virus abrogation of interleukin-3 dependence in a lymphoid cell line. Mol Cell Biol 6:4133–4135, 1986.
17. Cook WD, Metcalf D, Nicola NA, Burgess AW, Walker F: Malignant transformation of a growth factor-dependent myeloid cell line by abelson virus without evidence of an autocrine mechanism. Cell 41:677–683, 1985.
18. Shields A, Goff S, Paskind M, Otto G, Baltimore D: Structure of the abelson murine leukemia virus genome. Cell 18:955–962, 1979.
19. van de Ven WJ, Reynolds FH, Nalewaik RP, Stephenson JR: The nonstructural component of the Abelson murine leukemia virus polyprotein p120 is encoded by newly acquired genetic sequences. J Virol 32:1041–1045, 1979.
20. Witte ON, Rosenberg NE, Baltimore D: A normal cell protein cross-reactive to the major abelson murine leukaemia virus gene product. Nature 5730:396–398, 1979.
21. Goff SP, Gilboa E, Witte ON, Baltimore D: Structure of the abelson murine leukemia virus genome and the homologous cellular gene: Studies with cloned viral DNA. Cell 22:777–785, 1980.

22. Reddy EP, Smith MJ, Srinivasan A: Nucleotide sequence of Abelson murine leukemia virus genome: Structural similarity of its transforming gene product to other *onc* gene products with tyrosine-specific kinase activity. Proc Natl Acad Sci USA 80:3623–3627, 1983.
23. Bishop JM: Cellular oncogenes and retroviruses. Ann Rev Biochem 52:301–354, 1983.
24. Schultz A, Oroszlan S: Myristylation of *gag-onc* fusion proteins in mammalian transforming retroviruses. Virology 133:431–437, 1984.
25. Witte ON, Ponticelli A, Gifford A, Baltimore D, Rosenberg N, Elder J: Phosphorylation of the abelson murine leukemia virus transforming protein. J Virol 39:870–878, 1981.
26. Sefton BM, Hunter T, Raschke WC: Evidence that the Abelson virus protein function in vivo as a protein kinase that phosphorylates tyrosine. Proc Natl Acad Sci USA 78:1552–1556, 1981.
27. Witte ON, Goff S, Rosenberg N, Baltimore D: A transformation-defective mutant of abelson murine leukemia virus lacks protein kinase activity. Proc Natl Acad Sci USA 77:4993–4997.
28. Prywes R, Foulkes JG, Baltimore D: The minimum transforming region of v-*abl* is the segment encoding protein-tyrosine kinase. J Virol 54:114–122, 1985.
29. Engelman A, Rosenberg N: Isolation of temperature-sensitive Abelson virus mutants by site-directed mutagenesis. Proc Natl Acad Sci USA 84:8021–8025, 1987.
30. Kipreos E, Lee GJ, Wang JYJ: Isolation of temperature-sensitive tyrosine kinase mutants of v-*abl* oncogene by screening with antibodies for phosphotyrosine. Proc Natl Acad Sci USA 84:1345–1349, 1987.
31. Boss MA, Dreyfuss G, Baltimore D: Localization of the abelson murine leukemia virus protein in a detergent-insoluble subcellular matrix: Architecture of the protein. J Virol 40:472–481, 1981.
32. Rohrschneider LR, Najita LM: Detection of the v-abl gene product at cell-substratum contact sites in Abelson murine leukemia virus-transformed fibroblasts. J Virol 51:547–552, 1984.
33. Van Etten RA, Jackson P, Baltimore D: The mouse type IV c-*abl* gene product is a nuclear protein, and activation of transforming ability is associated with cytoplasmic localization. Cell 58:669–678, 1989.
34. Cross FR, Garber EA, Pellman D, Hanafusa H: A short sequence in the $p60^{src}$ N terminus is required for $p60^{src}$ myristylation and membrane association and for cell transformation. Mol Cell Biol 4:1834–1842, 1984.
35. Daley GQ, Van Etten RA, Jackson PJ, Bernards A, Baltimore D: Non-myristoylated Abl proteins transform a factor-dependent hematopoietic cell line. Mol Cell Biol 12:1864–1871, 1992.
36. Kamps MP, Buss JE, Sefton BM: Rous sarcoma virus transforming protein lacking myristic acid phosphorylates known polypeptide substrates without inducing transformation. Cell 45:105–112, 1986.
37. Green PL, Kaehler DA, McKearn J, Risser R: Substitution of the LTR of Abelson murine leukemia virus does not alter the cell type of virally induced tumors. Oncogene 2:383–392, 1988.
38. Daley GQ, Van Etten RA: Unpublished observations.
39. Besmer P, Hardy WD, Zuckerman EE, Bergold P, Lederman L, Snyder HW, Jr.: The Hardy-Zuckerman 2-FeSV, a new feline retrovirus with oncogene homology to Abelson-MuLV. Nature 303:825–828, 1983.
40. Bergold PJ, Blumenthal JA, D'Andrea E, Snyder HW, Lederman L, Silverstone A, Nguyen H, Besmer P: Nucleic acid sequence and oncogenic properties of the HZ2 feline sarcoma virus v-*abl* insert. J Virol 61:1193–1202, 1987.
41. Wang JYJ, Ledley F, Goff S, Lee R, Groner Y, Baltimore D: The mouse c-abl locus: Molecular cloning and characterization. Cell 36:349–356.
42. Bernards A, Rubin CM, Westbrook CA, Paskind M, Baltimore D: The first intron in the human c-*abl* gene is at least 200 kilobases long and is a target for translocations in chronic myelogenous leukemia. Mol Cell Biol 7:3231–3236, 1984.

43. Wang JYJ, Baltimore D: Cellular RNA homologous to the abelson murine leukemia virus transforming gene: Expression and relationship to the viral sequence. Mol Cell Biol 3:773–779, 1983.
44. Oppi C, Shore SK, Reddy EP: Nucleotide sequence of testis-derived c-*abl* cDNAs: Implications for testis-specific transcription and *abl* oncogene activation. Proc Natl Acad Sci USA 84:8200–8204, 1987.
45. Ben-Neriah Y, Bernards A, Paskind M, Daley GQ, Baltimore D: Alternative 5′ exons in c-*abl* mRNA. Cell 44:577–586, 1986.
46. Shtivelman E, Lifshitz B, Gale RP, Roe BA, Canaani E: Alternative splicing of RNAs transcribed from the human *abl* gene and from the *bcr-abl* fused gene. Cell 47:277–284, 1986.
47. Hanks SK, Quinn AM, Hunter T: The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. Science 241:42–52, 1988.
48. Kruh GD, King CR, Kraus MH, Popescu NC, Amsbaugh SC, McBride WO, Aaronson SA: A novel human gene closely related to the *abl* proto-oncogene. Science 234:1545–1548, 1986.
49. Kruh GD, Perego R, Miki T, Aaronson SA: The complete coding sequence of arg defines the Abelson subfamily of cytoplasmic tyrosine kinases. Proc Natl Acad Sci USA 87:5802–5806, 1990.
50. Henkemeyer MJ, Bennett RL, Gertler FB, Hoffmann FM: DNA sequence, structure, and tyrosine kinase activity of the *Drosophila melanogaster* abelson proto-oncogene homolog. Mol Cell Biol 8:843–853, 1988.
51. Gertler FB, Bennett RL, Clark MJ, Hoffmann FM: Drosophila *abl* tyrosine kinase in embryonic CNS axons: A role in axonogenesis is revealed through dosage-sensitive interactions with *disabled*. Cell 58:103–113, 1989.
52. Schwartzberg PL, Stall AM, Hardin JD, Bowdish KS, Humaran T, Boast S, Harbison ML, Robertson EJ, Goff SP: Mice homozygous for the *abl*^{m1} mutation show poor viability and depletion of selected B and T cell populations. Cell 65:1165–1176, 1991.
53. Tybulewicz VLJ, Crawford CE, Jackson PK, Mulligan RC: Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl protooncogene. Cell 65:1153–1164, 1991.
54. Towler DA, Eubanks SR, Towery DS, Adams SP, Glaser L: Amino-terminal processing of proteins by *N*-myristoylation. J Biol Chem 262:1030–1036, 1987.
55. Jackson P, Baltimore D: N-terminal mutations activate the leukemogenic potential of the myristoylated form of c-*abl*. EMBO J 8:449–456, 1989.
56. Shaw AS, Amrein KE, Hammond C, Stern DF, Sefton BM, Rose JK: The lck tyrosine protein kinase interacts with the cytoplasmic tail of the CD4 glycoprotein through its unique amino-terminal domain. Cell 59:627–636, 1989.
57. Resh MD: Specific and saturable binding of pp60$^{v\text{-}src}$ to plasma membranes: Evidence for a myristyl-*src* receptor. Cell 58:281–286, 1989.
58. Sadowski I, Stone JC, Pawson T: A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus P130$^{gag\text{-}fps}$. Mol Cell Biol 6:4396–4408, 1986.
59. Pawson T: Non-catalytic domains of cytoplasmic protein-tyrosine kinases: Regulatory elements in signal transduction. Oncogene 3:491–495, 1988.
60. Wang JYJ, Baltimore D: Localization of tyrosine kinase-coding region in v-*abl* oncogene by the expression of v-*abl*-encoded proteins in bacteria. J Biol Chem 260:64–71, 1985.
61. Koch C, Moran MF, Sadowski I, Pawson T: The common *src* homology region 2 domain of cytoplasmic signalling proteins is a positive effector of v-*fps* tyrosine kinase function. Mol Cell Biol 9:4131–4140, 1989.
62. DeClue JE, Martin GS: Linker insertion-deletion mutagenesis of the v-*src* gene: Isolation of host- and temperature-dependent mutants. J Virol 63:542–554, 1989.
63. Mayer BJ, Jackson PK, Baltimore D: High-affinity binding of the noncatalytic SH2

segment of the abl tyrosine kinase to tyrosine-phosphorylated cellular proteins. Proc Natl Acad Sci USA 88:627–631, 1990.
64. Moran MF, Koch DA, Ellis C, England L, Martin GS, Pawson T: Src homology region 2 domains direct protein-protein interactions in signal transduction. Proc Natl Acad Sci USA 87:8622–8626, 1990.
65. Cantley LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R, Soltoff S: Oncogenes and signal transduction. Cell 64:281–302, 1991.
66. Pendergast AM, Traugh JA, Witte ON: Normal cellular and transformation-associated *abl* proteins share common sites for protein kinase C phosphorylation. Mol Cell Biol 7:4280–4289, 1987.
67. Kipreos ET, Wang JYJ: Differential phosphorylation of c-Abl in cell cycle determined by *cdc*2 kinase and phosphatase activity. Science 248:217–220, 1990.
68. Van Etten R, Jackson P, Baltimore D: Unpublished results.
69. Prywes R, Foulkes JG, Rosenberg N, Baltimore D: Sequences of the A-MuLV protein needed for fibroblast and lymphoid cell transformation. Cell 34:569–579, 1983.
70. Watanabe SM, Witte ON: Site-directed deletions of Abelson murine leukemia virus define 3′ sequences essential for transformation and lethality. J Virol 45:1028–1036, 1983.
71. Ziegler SF, Whitlock CA, Goff SP, Gifford A, Witte ON: Lethal effect of the abelson murine leukemia virus transforming gene product. Cell 27:477–486, 1981.
72. Goff SP, Tabin CJ, Wang JY-J, Weinberg R, Baltimore D: Transfection of fibroblasts by cloned abelson murine leukemia virus DNA and recovery of transmissible virus by recombination with helper virus. J Virol 41:271–285, 1982.
73. Jackson P, Van Etten RA, Baltimore D: Unpublished results.
74. Franz WM, Berger P, Wang JYJ: Deletion of an N-terminal regulatory domain of the c-*abl* tyrosine kinase activates its oncogenic potential. EMBO J 8:137–147, 1989.
75. Jackson PK, Paskind M, Baltimore D: Activation of transforming ability of c-abl by point mutation in the tyrosine kinase domain. Submitted.
76. Shore SK, Bogart SL, Reddy EP: Activation of murine c-abl protooncogene: Effect of a point mutation on oncogenic activation. Proc Natl Acad Sci USA 87:6502–6506, 1990.
77. Jackson P, Van Etten RA, Daley GQ, Baltimore D: N-terminal mutations can activate c-abl independent of SH3 mutation. Submitted, 1991.
78. Piwinica-worms H, Saunders K, Roberts T, Smith A, Cheng S: Tyrosine phosphorylation regulates the biochemical and biological properties of pp60c-src. Cell 49:75–82, 1987.
79. Groffen J, Heisterkamp N, Reynolds FH, Jr., Stephenson JR: Homology between phosphotyrosine acceptor site of human c-*abl* and viral oncogene products. Nature 304:167–169, 1983.
80. Jackson P: Unpublished results.
81. Ponticelli AS, Whitlock CA, Rosenberg N, Witte ON: In vivo tyrosine phosphorylations of the abelson virus transforming protein are absent in its normal cellular homolog. Cell 29:953–960, 1982.
82. Pendergast AM, Muller AJ, Havlik MH, Clark R, McCormick F, Witte ON: Evidence for regulation of the human ABL tyrosine kinase by a cellular inhibitor. Proc Natl Acad Sci USA 88:5927–5931, 1991.
83. Konopka JB, Witte ON: Detection of c-*abl* tyrosine kinase activity in vitro permits direct comparison of normal and altered *abl* gene products. Mol Cell Biol 5:3116–3123, 1985.
84. Lugo TG, Pendergast A, Muller AJ, Witte ON: Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. Science 247:1079–1082, 1990.
85. McWhirter JR, Wang JYJ: Activation of tyrosine kinase and microfilament-binding functions of c-*abl* by bcr sequences in *bcr/abl* fusion proteins. Mol Cell Biol 11:1785–1792, 1991.
86. Sporn M, Todaro G: Autocrine secretion and malignant transformation of cells. N Engl J Med 303:878–880, 1980.
87. Twardzik DR, Todaro GJ, Marquardt H, Reynolds FH, Jr., Stephenson JR:

Transformation induced by abelson murine leukemia virus involves production of a polypeptide growth factor. Science 216:894–897, 1982.
88. Gebhardt A, Bell JC, Foulkes JG: Abelson transformed fibroblasts lacking the EGF receptor are not tumourigenic in nude mice. EMBO J 5:2191–2195, 1986.
89. Hunter T, Cooper JA: Viral oncogenes and tyrosine phosphorylation. In: The Enzymes. Academic Press, New York, 1986, pp 191–246.
90. Varticovski L, Daley G, Jackson P, Baltimore D, Cantley L: Activation of PI 3-kinase in cells expressing abl oncogene variants. Mol Cell Biol 11:1107–1113, 1991.
91. Moran MF, Polakis P, McCormick F, Pawson T, Ellis C: Protein-tyrosine kinases regulate the phosphorylation, protein interactions, subcellular distribution, and activity of $p21^{ras}$ GTPase-activating protein. Mol Cell Biol 11:1804–1812, 1991.
92. Champlin RE, Golde DW: Chronic myelogenous leukemia: Recent advances. Blood 65:1039–1047, 1985.
93. Nowell PC, Hungerford DA: A minute chromosome in human chronic granulocytic leukemia. Science 132:1197–1200, 1960.
94. Rowley JD: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 243:290–293, 1973.
95. Whang J, Frei III E, Tjio JH, Carbone PP, Brecher G: The distribution of the Philadelphia chromosome in patients with chronic myelogenous leukemia. Blood 22:664–673, 1963.
96. Tough IM, Jacobs PA, Court-Brown WM, Baikie AG, Williamson ERD: Cytogenetic studies on bone-marrow in chronic myeloid leukaemia. Lancet 1:844–846, 1963.
97. Allouche M, Bourinbaiar A, Georgoulias V: T-cell lineage involvement in lymphoid blast crisis of chronic myeloid leukemia. Blood 66:1155–1161, 1985.
98. Kurzrock R, Gutterman J, Talpaz M: The molecular genetics of Philadelphia chromosome-positive leukemias. N Engl J Med 319:990–998.
99. Clark SS, Crist WM, Witte ON: Molecular pathogenesis of Ph-positive leukemias. Ann Rev Med 40:113–122, 1988.
100. Daley GQ, Ben-Neriah Y: Implicating the *bcr/abl* gene in the pathogenesis of Philadelphia chromosome-positive human leukemia. In: Advances in Cancer Research. Academic Press, San Diego, 1991, pp 151–184.
101. de Klein A, van Kessel AG, Grosveld G, Bartram CR, Hagemeier A, Bootsma D, Spurr NK, Heisterkamp N, Groffen J, Stephenson JR: A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukemia. Nature 300:765–767, 1982.
102. Heisterkamp N, Stephenson JR, Groffen J, Hansen PF, de Klein A, Bartram CR, Grosveld G: Localization of the c-abl oncogene adjacent to a translocation break point in chronic myelocytic leukaemia. Nature 306:239–242, 1983.
103. Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, Grosveld G: Philadelphia chromosomal breakpoints are clustered within a limited region, *bcr*, on chromosome 22. Cell 36:93–99, 1984.
104. Collins S, Coleman H, Groudine M: Expression of *bcr* and *bcr-abl* fusion transcripts in normal and leukemic cells. Mol Cell Biol 7:2870–2876, 1987.
105. Konopka JB, Watanabe SM, Witte ON: An alteration of the human c-*abl* protein in K562 leukemia cells unmasks associated tyrosine kinase activity. Cell 37:1035–1042, 1984.
106. Shtivelman E, Lifshitz B, Gale RP, Canaani E: Fused transcript of *abl* and *bcr* genes in chronic myelogenous leukaemia. Nature 315:550–554, 1985.
107. Ben-Neriah Y, Daley GQ, Mes-Masson A-M, Witte ON, Baltimore D: The chronic myelogenous leukemia-specific P210 protein is the product of the *bcr/abl* hybrid gene. Science 233:212–214, 1986.
108. Clark SS, McLaughlin J, Crist WM, Champlin R, Witte ON: Unique forms of the *abl* tyrosine kinase distinguish Ph^1-positive CML from Ph^1-positive ALL. Science 235:285–87, 1987.
109. Hermans A, Heisterkamp N, von Lindern M, van Baal S, Meijer D, van der Plas D,

Wiedemann LM, Groffen J, Bootsma D, Grosveld G: Unique fusion of bcr and c-abl genes in Philadelphia chromosome positive acute lymphoblastic leukemia. Cell 51:33–40, 1987.
110. Kurzrock R, Shtalrid M, Talpaz M, Kloetzer WS, Gutterman JU: Expression of c-*abl* in Philadelphia-positive acute myelogenous leukemia. Blood 70:1584–1588, 1987.
111. Selleri L, von Lindern M, Hermans A, Meijer D, Torelli G, Grosveld G: Chronic myeloid leukemia may be associated with several bcr-abl transcripts including the acute lymphoid leukemia-type 7 kb transcript. Blood 75:1146–1153, 1990.
112. Dreazen O, Canaani E, Gale RP: Molecular biology of chronic myelogenous leukemia. Semin Hematol 25:35–49, 1988.
113. Chen SJ, Flandrin G, Daniel MT, Valensi F, Baranger L, Grausz D, Bernheim A, Chen Z, Sigaux F, Berger R: Philadelphia-positive leukemia: Lineage promiscuity and inconsistently rearranged breakpoint cluster region. Leukemia 2:261–273, 1988.
114. Hirsch-Ginsberg C, Childs C, Chang K-S, Beran M, Cork A, Reuben J, Freireich EJ, Chang LCM, Bollum FJ, Trujillo J, Stass SA: Phenotypic and molecular heterogeneity in Philadelphia chromosome-positive acute leukemia. Blood 71:186–195, 1988.
115. Stam K, Heisterkamp N, Reynolds FH, Jr., Groffen J: Evidence that the *phl* gene encodes a 160,000-dalton phosphoprotein with associated kinase activity. Mol Cell Biol 7:1955–1960, 1987.
116. Timmons MS, Witte ON: Structural characterization of the *bcr* gene product. Oncogene 4:559–567, 1989.
117. Hariharan IK, Adams JA: cDNA sequence for human *bcr*, the gene that translocates to the *abl* oncogene in chronic myeloid leukaemia. EMBO J 6:115–119, 1987.
118. Diekmann D, Brill S, Garrett MD, Totty N, Hsuan J, Monfries C, Hall C, Lim L, Hall A: *Bcr* encodes a GTPase-activating protein for $p21^{rac}$. Nature 351:400–402, 1991.
119. Muller AJ, Young JC, Pendergast A, Pondel M, Landau NR, Littman DR, Witte ON: BCR first exon sequences specifically activate the BCR/ABL tyrosine kinase oncogene of Philadelphia chromosome-positive leukemias. Mol Cell Biol 11:1785–1792, 1991.
120. Pendergast AM, Muller AJ, Havlik MH, Maru Y, Witte ON: BCR sequences essential for transformation by the *BCR-ABL* oncogene bind to the ABL SH2 regulatory domain in a non-phosphotyrosine-dependent manner. Cell 66:161–171, 1991.
121. Hariharan IK, Adams JM, Cory S: bcr-abl oncogene renders myeloid cell line factor independent: Potential autocrine mechanism in chronic myeloid leukemia. Oncogene Res 3:387–399, 1988.
122. Daley G, Baltimore D: Transformation of an interleukin 3-dependent hematopoietic cell line by the chronic myelogenous leukemia-specific P210 *bcr/abl* protein. Proc Natl Acad Sci USA 85:9312–9316, 1988.
123. Laneuville P, Heisterkamp N, Groffen J: Expression of the chronic myelogenous leukemia-associated p210 *bcr/abl* oncoprotein in a murine IL-3 dependent myeloid cell line. Oncogene 6:275–282, 1991.
124. Daley GQ, McLaughlin J, Witte ON, Baltimore D: The CML-specific P210 *bcr/abl* protein, unlike v-*abl*, does not transform NIH/3T3 fibroblasts. Science 237:532–535, 1987.
125. Lugo TG, Witte ON: The *bcr-abl* oncogene transforms rat-1 cells and cooperates with v-*myc*. Mol Cell Biol 9:1263–1270, 1989.
126. McLaughlin J, Chianese E, Witte ON: In vitro transformation of immature hematopoietic cells by the P210 *bcr/abl* oncogene product of the Philadelphia chromosome. Proc Natl Acad Sci USA 84:6558–6562, 1987.
127. Young JC, Witte ON: Selective transformation of primitive lymphoid cells by the *bcr/abl* oncogene expressed in long-term lymphoid or myeloid cultures. Mol Cell Biol 8:4079–4087, 1988.
128. McLaughlin J, Chianese E, Witte ON: Alternative forms of the *bcr/abl* oncogene have quantitatively different potencies for stimulation of immature lymphoid cells. Mol Cell Biol 9:1866–1874, 1989.
129. Scherle PA, Dorshkind K, Witte ON: Clonal lymphoid progenitor cell lines expressing the

BCR/ABL oncogene retain full differentiative function. Proc Natl Acad Sci USA 87:1908–1912, 1990.
130. Rosenbaum H, Harris AW, Bath ML, McNeall J, Webb E, Adams JM, Cory S: An E(mu)-v-abl transgene elicits plasmacytomas in concert with an activated myc gene. EMBO J 9:897–905, 1990.
131. Heisterkamp N, Jenster G, ten Hoeve J, Zovich D, Pattengale PK, Groffen J: Acute leukemia in bcr/abl transgenic mice. Nature 344:251–253, 1990.
132. Hariharan IK, Harris AW, Crawford M, Abud H, Webb E, Cory S, Adams JM: A *bcr*-v-*abl* oncogene induces lymphomas in transgenic mice. Mol Cell Biol 9:2798–2805, 1989.
133. Grosveld G: Personal communication.
134. Elefanty AG, Hariharan IK, Cory S: *bcr-abl*, the hallmark of chronic myeloid leukemia in man, induces multiple hematopoietic neoplasms in mice. EMBO J 9:1069–1078, 1990.
135. Daley GQ, Van Etten RA, Baltimore D: Induction of chronic myelogenous leukemia in mice by the P210 *bcr/abl* gene of the Philadelphia chromosome. Science 247:824–830, 1990.
136. Daley GQ, Van Etten RA, Baltimore D: Blast crisis in a murine model of chronic myelogenous leukemia. Proc Natl Acad Sci USA 88:11335–11338, 1991.
137. Kelliher MA, McLaughlin J, Witte ON, Rosenberg N: Induction of a chronic myelogenous leukemia-like syndrome in mice with v-abl and bcr/abl. Proc Natl Acad Sci USA 87:6649–6653, 1990.
138. Scott ML, Van Etten RA, Daley GQ, Baltimore D: v-*abl* causes hematopoietic disease distinct from that caused by *bcr-abl*. Proc Natl Acad Sci USA 88:6506–6510, 1991.
139. Chung S-W, Wong PMC, Durkin H, Wu Y-S, Petersen J: Leukemia initiated by hemopoietic stem cells expressing the v-*abl* oncogene. Proc Natl Acad Sci USA 88:1585–1589, 1991.
140. Kelliher M, Knott A, McLaughlin J, Witte ON, Rosenberg N: Differences in oncogenic potency but not target cell specificity distinguish the two forms of the *BCR/ABL* oncogene. Mol Cell Biol 11:4710–4716, 1991.
141. Van Etten RA, Daley GQ, in preparation.

9. The biological and clinical roles of increased insulin receptors in human breast cancer

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Introduction

Both steroid hormones and peptide growth factors are known to control the growth and progression of breast cancers [1,2]. Human and animal breast carcinomas have receptors for steroid and peptide hormones, and both in vitro and in vivo studies have indicated that tumor proliferation rate and overall growth are dependent on these ligand-receptor systems [3,4]. Approximately one third to one half of all breast cancer cases show estrogen-dependent growth [3], and clinical studies have indicated that patients whose tumors have estrogen or progesterone receptors (ER, PR) have biologically less aggressive tumors and live longer than those patients whose tumors lack ER or PR [1,5]. However, some receptor-positive breast cancers are not normally responsive to steroid hormone regulation, behave much more aggressively, and reduce patient survival [5,6]. One explanation for this discrepancy between steroid-receptor positivity and tumor behavior is that the tumor may lose certain intracellular mechanisms that mediate hormonal growth control. A second explanation is that these receptor-positive tumors are heterogeneous and contain subpopulations of receptor-negative cells capable of more aggressive behavior. A third possibility is that the more aggressive tumor cell behavior is derived from the influence of other hormones, such as polypeptide hormones and growth factors. These latter factors may play autocrine and paracrine roles in determining aggressive breast tumor behavior, including a more rapid tumor proliferation rate and steroid hormone resistance. For this reason it is important to quantitate tumor receptor levels in concert with histological analysis and other techniques to identify the cells in tumors that contain receptors for steroid hormones and polypeptide growth factors.

Recent investigations have focused on growth factors and receptors that are members of the tyrosine kinase family [7]. A number of growth factors stimulate cellular mitogenesis by interacting with a family of cell surface receptors that possess an intrinsic ligand-sensitive protein tyrosine kinase activity. It is believed that tyrosine phosphorylation of key cellular proteins

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initiates changes in cell growth. Tyrosine kinase receptors are typically composed of an extracellular ligand binding domain that is linked to a cytoplasmically oriented catalytic domain, which not only transduces the growth factor or hormonal signal but also generates mitogenic second messengers. There are now four subclasses of tyrosine kinase receptors [7]. Subclass I includes the epidermal growth factor receptor (EGF-R), which is activated by the ligands EGF and transforming growth factor α (TGF-α) and the closely related HER-2/*neu* receptor. Subclass II receptors include the insulin receptor (IR) and the closely related insulin-like growth factor-I receptor (IGF-I-R), which are activated by insulin and IGF-I, respectively. Subclass III receptors include the platelet-derived growth factor receptor (PDGF-R) and the colony stimulating factor receptor (CSF-I-R). Subclass IV receptors include the fibroblast growth factor receptor (FGF-R) and its relatives [8]. In addition to these proto-oncogene encoded tyrosine kinase receptors, there are also receptor-derived viral oncogene products. V-*erb* B is derived from the EGF-R, and v-*fms* from the CSF-I-R. In general, these viral oncogene products differ from their normal receptor counterparts in that they have either amino acid deletions or substitutions that enable them to possess ligand-independent (and thus constitutively activated) tyrosine kinase activity.

Several mechanisms have been suggested [7] by which receptor tyrosine kinases and their derived oncogenes are believed to be implicated in tumor formation. These include increased receptor kinase content (secondary to gene amplification or overexpression) and inappropriate enzymatic activity (e.g., mutant protein) with otherwise normal levels of the receptor protein. In a recent survey, breast tumors were commonly found to have high tyrosine kinase activity that correlated with poor patient prognosis; however, the specific kinases involved were not identified in this study [9]. In breast cancers, the most commonly activated subclasses of tyrosine kinase receptors are EGF-R, HER-2/*neu*, and IGF-I-R. Gene amplification and/or overexpression has been reported for HER-2/*neu*, whereas overexpression without amplification is characteristic of EGF-R [7].

The largest number of clinical studies in breast cancer relate to the HER-2/*neu* receptor [10,11]. This proto-oncogene product if overexpressed in approximately 30% of breast cancers [10,11]; and in cases that are lymph node positive, there is a negative correlation between gene amplification (or protein overexpression) in the tumor and patient survival [10,11]. These cases do not constitute the majority of breast tumors with elevated tyrosine kinase activity, and some, but not all, of those tumors overexpressing tyrosine kinase activity may also overexpress the EGF-R (found in 30–40% of human breast tumors), which is also a predictor for poor patient survival [10,11]. The role of other tyrosine kinase receptors in breast cancer needs to be elucidated. Our studies have focused on the insulin receptor (a tyrosine kinase receptor of subclass II), its characteristics, and its role in human breast cancer.

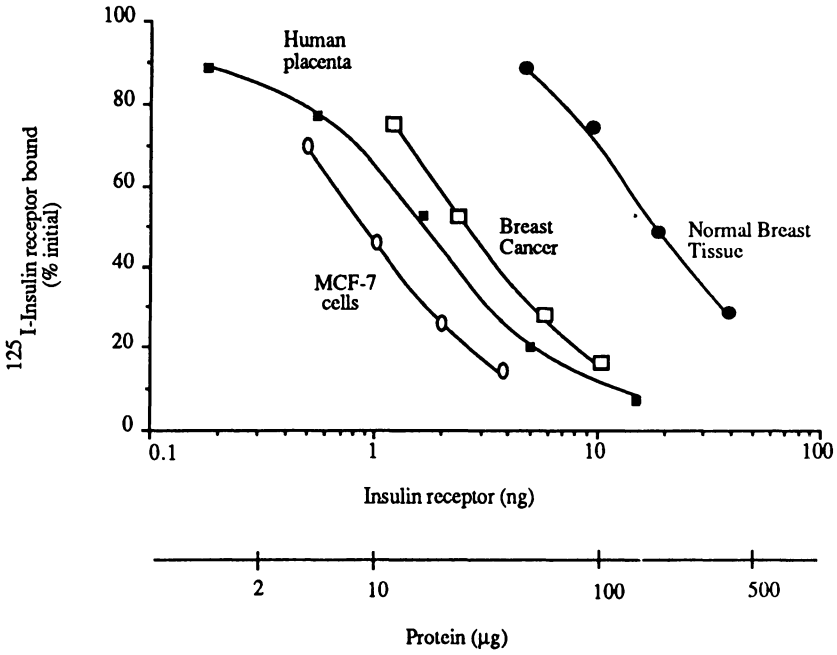

Figure 1. Competition-inhibition curves for the purified placenta insulin receptor standard (■) and extracts of human MCF-7 breast cancer cells (○), breast cancer tissue (□), and normal breast tissue (●) on the binding of labeled insulin. (From Papa et al. [23] with permission.)

Insulin receptor content in human breast cancer specimens

Insulin regulates the growth and metabolism of animal breast cancer cells both in vivo and in vitro [12–14] and human breast cells in vitro [13,15–18]. Moreover, several human breast cancer cell lines in permanent culture have specific, high-affinity receptors for insulin [19,20], and the presence of insulin receptors has been reported in frozen specimens of human breast carcinomas [20,21]. For several reasons, however, the importance of these observations was unclear. First, a quantitative comparative analysis between the number of receptors in tumor cells vs. normal mammary gland tissue had not been carried out. Second, in human cancers correlations between insulin receptor content and established prognostic parameters of cancer evolution, including estrogen and progesterone receptor content, and tumor size and grading had not been made.

We have recently employed a new, sensitive, and specific radioimmunoassay [22] for the direct measurement of insulin receptors [23] in surgical specimens of breast cancers (Fig. 1). In 159 specimens the insulin receptor content averaged 6.15 ± 3.69 ng/0.1 mg protein (Fig. 2). This value was more than sixfold higher than the mean value found in both 27 normal breast tissues obtained at total mastectomy (0.95 + 0.68, $p < 0.001$) and in

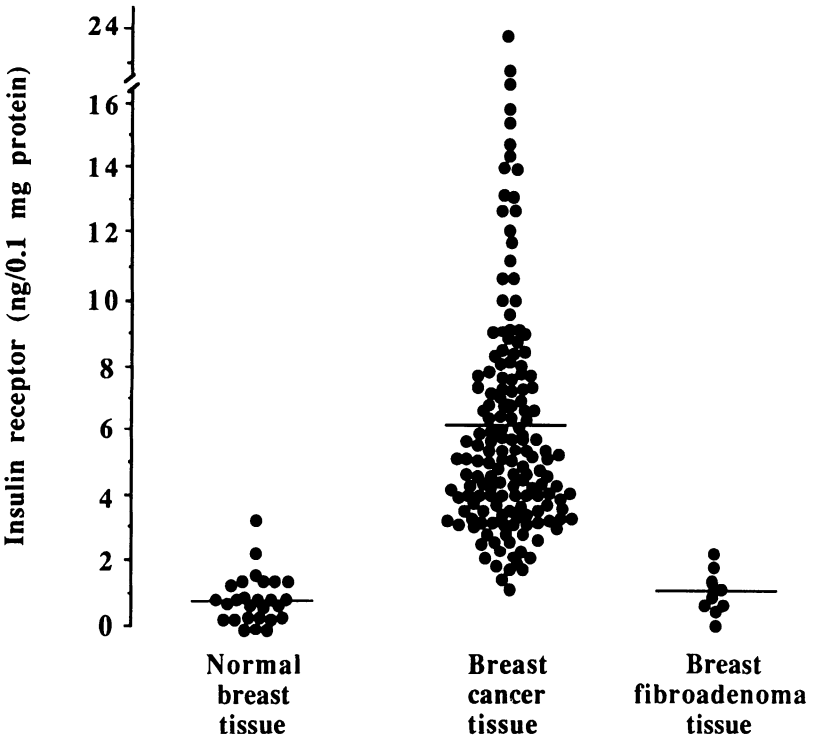

Figure 2. Insulin receptor content in normal breast tissue, breast cancer, and breast fibroadenoma specimens. (From Papa et al. [23] with permission.)

six normal specimens obtained from reduction mammoplasty (0.84 ± 0.78, $p < 0.001$). The insulin receptor content in breast cancer tissues was also higher than in any normal tissue investigated, including liver. Immunostaining of the specimens revealed that the insulin receptor content was high in malignant epithelial cells (Fig. 3), but not in stromal and inflammatory cells.

Statistical analysis revealed that the insulin receptor content of the tumors correlated positively with tumor size ($p = 0.014$), histological grading ($p = 0.030$), and the estrogen receptor content ($p = 0.035$). There were no significant correlations between insulin receptor content and the age, body weight, menopausal status, and nodal involvement of the patients. These studies indicate, therefore, that the insulin receptor content is high in human breast cancers. The correlation between IR content and some clinical and biological characteristics of breast cancers raise the possibility that the insulin receptor may have a role in the biology of these tumors.

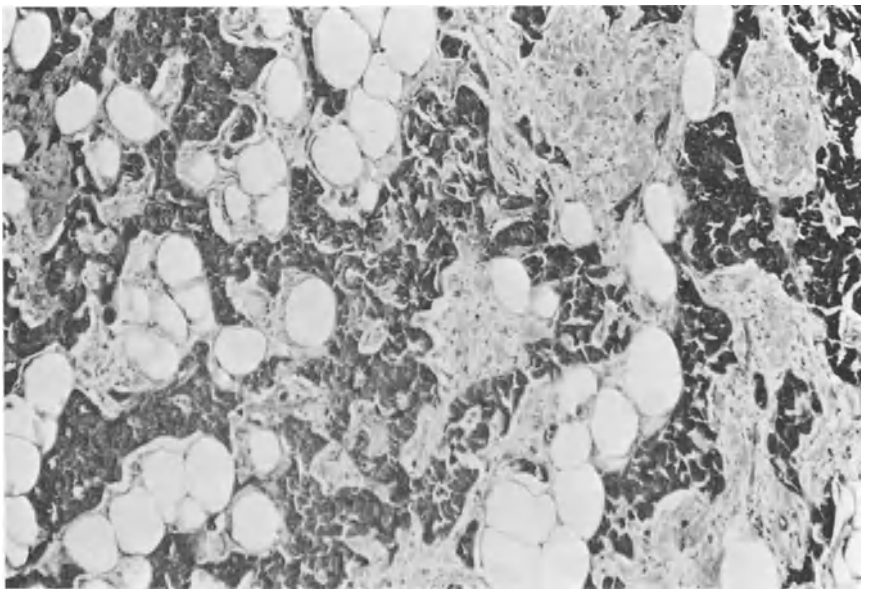

Figure 3. Representative sections of a ductal breast cancer. Indirect immunoperoxidase staining of the insulin receptor with insulin receptor monoclonal antibody. Specific staining, with various intensity, is located only in the cytoplasm of malignant epithelial cells, while stromal and other cells are not stained. Some heterogeneity in the intensity of malignant cell staining is evident at higher magnifications. (From Papa et al. [23] with permission.)

Insulin receptor structure and function in human breast cancer specimens

We next characterized the structure and function of insulin receptors isolated from 10 human breast cancer specimens. In both cancer and normal breast tissues, insulin receptor mRNA consisted of two major species of approximately 11.0 and 8.5 kb. The size of the insulin receptor alpha subunit was determined by ^{125}I-insulin crosslinking followed by immunoprecipitation and polyacrylamide gel electrophoresis; a value of 135 kDa was observed for receptors from both breast cancer and normal breast tissues (Fig. 4). The functional binding ability of insulin receptors from breast cancer tissues was slightly lower as compared to normal breast cancer tissues (% B/T per nanogroun of insulin receptor = 2.22 ± 0.50 vs. 2.96 ± 0.49, mean ± SEM). The concentration of insulin that caused half-maximal inhibition of ^{125}I-insulin binding was similar for both breast cancer tissue and normal breast tissue.

The size of the insulin receptor beta subunit molecular weight as determined by insulin receptor autophosphorylation was 95 kDa. Basal and maximal insulin (100 nM) stimulated receptor receptor autophosphorylation was similar in both breast cancer and normal breast tissues (Fig. 5). How-

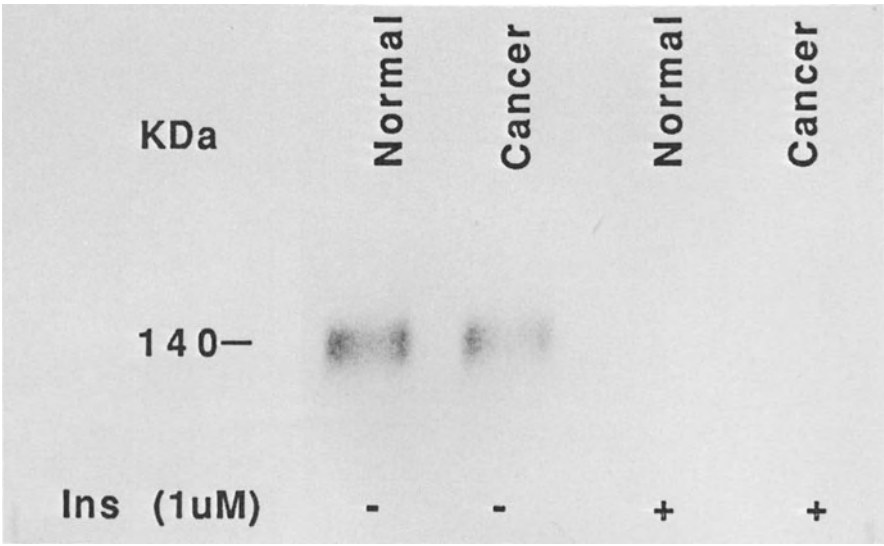

Figure 4. ^{124}I-insulin-receptor crosslinking to breast cancer specimens. WGA purified insulin receptor from normal and malignant tissues were incubated with ^{125}I-insulin in the absence or the presence of 1 μM unlabelled insulin. Next the ^{125}I-insulin-receptor complex was crosslinked with DSS, immunoprecipitated, and subject to PAGE.

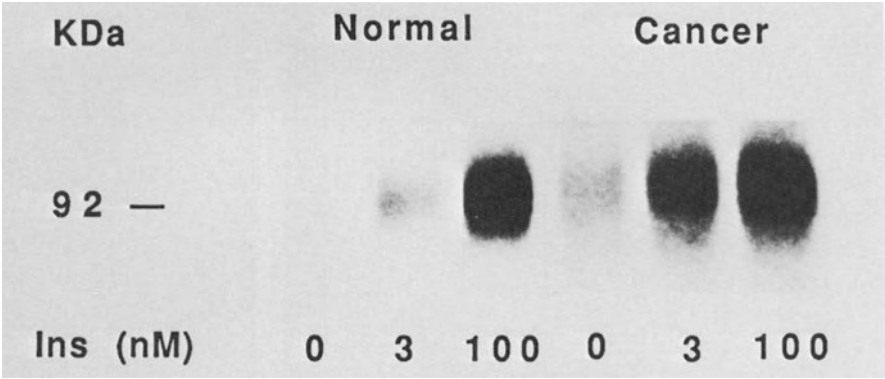

Figure 5. Insulin receptor autophosphorylation. WGA purified insulin receptors from normal and cancer tissues incubated in the absence or the presence of either 3 or 100 nM insulin for 60 minutes at 20°C. Next γP^{32}-ATP (1 μM) was added and the mixture was incubated for additional 30 minutes at 20°C. Insulin receptors were then immunoprecipitated by protein A-sepharose, boiled in the presence of 100 mM DTT, subjected to PAGE, and autoradiographed.

Table 1. Insulin receptor content in cultured breast cell lines

Cells	Insulin receptor content ($ng/10^6$ cells)
MCF-7	28.5 ± 4.5
ZR-75-1	17.5 ± 1.9
T-47D	4.8 ± 0.84
184B5	5.3 ± 1.7
184	4.1 ± 0.22

ever, insulin receptors from breast cancer tissues had a slightly lower basal tyrosine-kinase activity using the substrate poly(Glu-Tyr)4:1. The maximal insulin effect, expressed as the percent increase over basal value, was similar in insulin receptors from breast cancer and normal breast tissues (150 ± 10 vs. 142 ± 11, respectively).

In conclusion, breast cancer tissues have structurally and functionally normal insulin receptors. Since these receptors are overexpressed in breast cancer tissues, it is possible that, in vivo, insulin, by stimulating their tyrosine kinase activity, may play a role in the biology of certain breast cancers.

Insulin receptors in human breast cancer cell lines

In other to further understand the role of the insulin receptor in breast cancer, insulin receptor expression and function were characterized in three human breast cancer cell lines — MCF-7, ZR-75-1, and T-47D — and were compared to a nonmalignant human breast epithelial cell line, 184B5. Insulin receptor content, measured by radioimmunoassay, was fivefold and threefold elevated in MCF-7 and ZR-75-1 breast cancer cell lines, respectively, when compared to the nonmalignant epithelial cell line, 184B5 and a primary culture of breast epithelial cells 184 (Table 1). In contrast, the insulin receptor content of T-47D cells was not increased.

The increase in insulin receptor content in MCF-7 and ZR-751 cells was not due to amplification of the insulin receptor gene. Also, total insulin receptor mRNA content was not increased in breast cancer cells in comparison to nonmalignantly transformed 184B5 breast epithelial cells (Fig. 6). However, significant differences in the content of receptor mRNA species were observed. These observations raise the possibility that the observed increase in insulin receptor protein content in certain breast cancer cell lines is due to changes in either mRNA quality, enhanced mRNA translation, or enhanced protein stability.

The insulin receptors in the breast cancer cell lines were functional and receptor function correlated with receptor content: (1) In all cell lines,

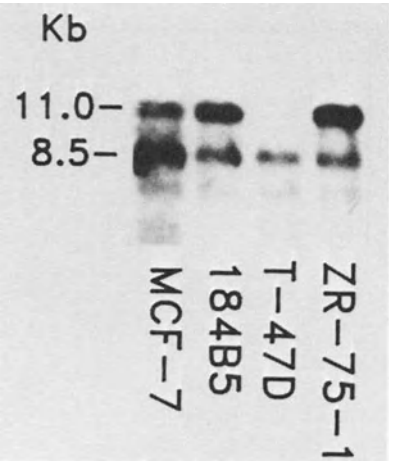

Figure 6. Northern blot analysis of insulin receptor mRNA in breast cell lines. Poly $(A)^+$ RNA was prepared from each cell line (8 μg) and was subjected to agarose gel electrophoresis, followed by transfer to nitrocellulose filters and hybridization with labeled insulin receptor cDNA.

high-affinity insulin binding was detected, and, in concert with the insulin receptor radioimmunoassay data, binding capacity was highest in MCF-7 and then ZR-75-1 cells. (2) In all cell lines, insulin stimulated insulin receptor tyrosine kinase activity. In concert with insulin receptor content and binding data, the greatest effects were observed in MCF-7 cells. (3) In all cell lines, insulin stimulated cell growth at concentrations of 1 nM or less. The effect of insulin was greater in MCF-7 cells, the cell line having the greatest insulin receptor expression and insulin receptor tyrosine kinase activity (Fig. 7). These studies thus demonstrated that certain human breast cancer cell lines, like human breast cancers in vivo, have enhanced expression of functional insulin receptors; moreover, like breast cancers in vivo, insulin receptor expression and function in breast cancer cell lines is heterogeneous. The study of human breast cancer cells in vitro, therefore, could provide important insights into hormonal regulation of breast tumor growth in vivo.

Progestin regulation of insulin receptor in human breast cancer cell

The effects of progesterone on the growth of breast carcinoma cells is still controversial. Thus we investigated the effect of progestins on insulin receptor gene expression and insulin action in human breast cancer cells [24]. Treatment of T47D cells with the synthetic progestin R5020 induced a time- and dose-dependent increase in insulin receptor content as measured by both ligand-binding studies and radioimmunoassay (Fig. 8). Binding was

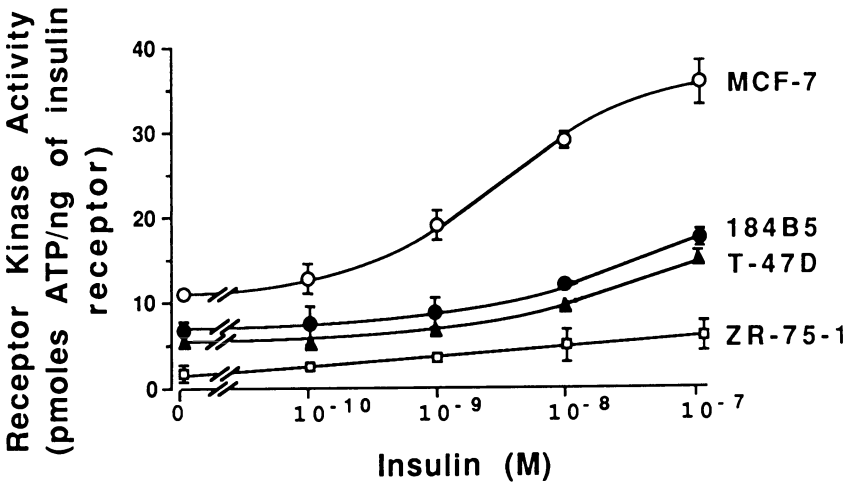

Figure 7. Mitogenic effect of insulin in nonmalignant human breast epithelial and human breast cancer cell lines. Monolayer cell cultures were incubated in serum-free medium with insulin at the indicated concentrations for 5 days. Then cellular DNA was measured.

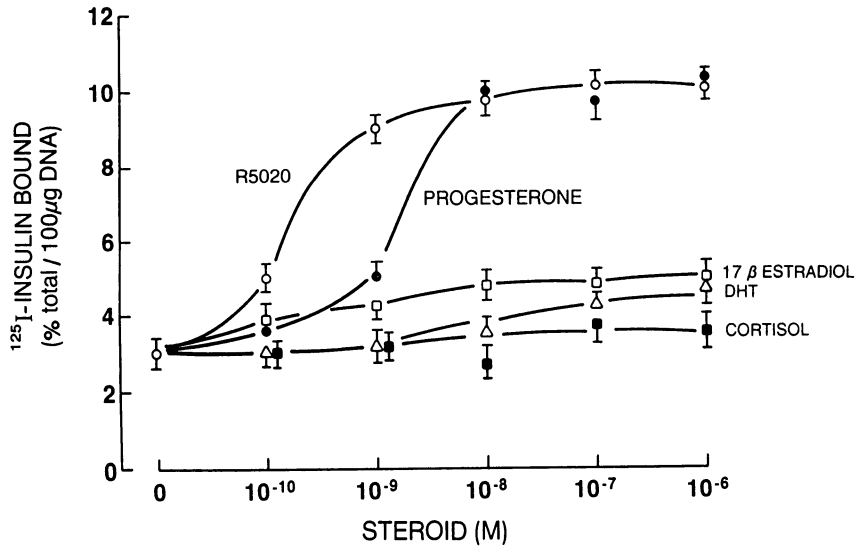

Figure 8. Influence of steroid hormones on ^{125}I-insulin binding to T47D cells. Cells were grown for 48 hours in the absence of steroid hormones and then the medium was replaced with medium containing the various steroid hormones at the concentrations shown. After 4 days of treatment, cells were harvested and specific ^{125}I-insulin binding was measured. DHT = dihydrotestosterone. All data were normalized to DNA content. Points represent the means of three separate experiments performed with triplicate determinations; bars, ±SE. (From Papa et al. [24] with permission.)

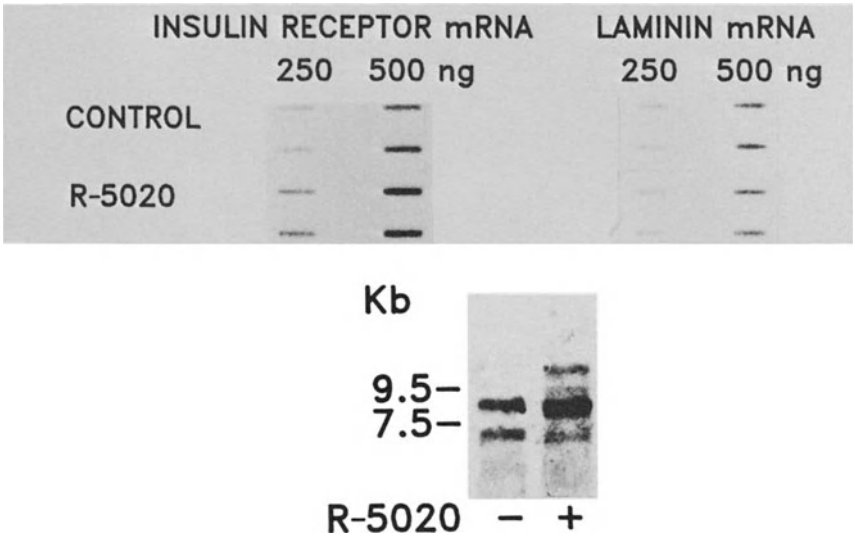

Figure 9. Effect of R5020 on insulin receptor mRNA. T47D cells were grown for 2 days without R5020 and then incubated 4 days in the absence or presence of 100 nM R5020. A: Slot blot of poly $(A)^+$ RNA from control and R5020-treated cells. Filters were probed with either labeled insulin receptor cDNA or labeled A-lamin cDNA. B: Northern blot of insulin receptor poly $(A)^+$ RNA from control (−) and R5020-treated (+) cells. (From Papa et al. [24] with permission.)

half-maximally increased at 300 pM R5020, and a maximal increase was reached after 4 days of treatment. Progesterone was 10-fold less potent than R5020, while 17 β-estradiol and dihydrotestosterone had minimal effects. Cortisol had no effect on insulin receptor levels. Progestin treatment both increased insulin receptor mRNA levels (Fig. 9) and altered the relative distribution of the multiple insulin receptor mRNA transcripts. In order to study the functional significance of the increased insulin receptor levels, we incubated T47D cells with progesterone and then treated them with insulin. Insulin alone had a small effect on cell growth; however, the effect of insulin was markedly potentiated by progesterone treatment. Similar effects of progestins on insulin-stimulated cell growth were seen with the MCF-7 cell line (Fig. 10). These studies in T47D and MCF-7 cells demonstrate, therefore, that insulin receptor expression in these breast cancer cells is under the regulation of progestins and raise the possibility that progestin—insulin interactions may regulate breast cancer cell growth in vivo.

Role of the insulin receptor in malignant transformation

To investigate the role of the insulin receptor in cancer we studied whether overexpression of the insulin receptor results in altered cell growth [25].

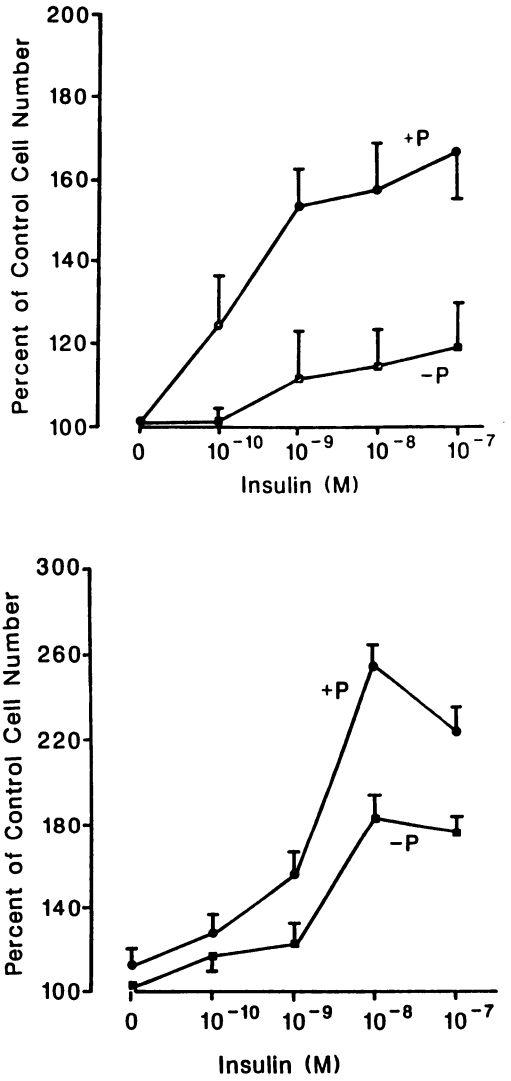

Figure 10. Effect of progesterone treatment on insulin-stimulated cell growth in breast cancer cells. Top: T47D cells Bottom: MCF-7 cells. (From Papa et al. [24] with permission.)

We used NIH 3T3 cells transfected with a bovine papilloma virus/insulin receptor cDNA construct (3T2/HIR). These cells expressed high numbers of insulin receptors (631.0 ± 16.7 ng receptors/10^6 cells). Insulin significantly stimulated the growth of 3T3/HIR cells maintained in serum-free medium (Fig. 11). Moreover, in these cells insulin induced marked phenotypic changes, including alterations in cell shape, loss of contact inhibition, and focal growth (Fig. 12). In contrast to 3T3/HIR cells, insulin was without effect in either wild-type 3T3 cells (3T3/wt), 3T3 cells transfected with

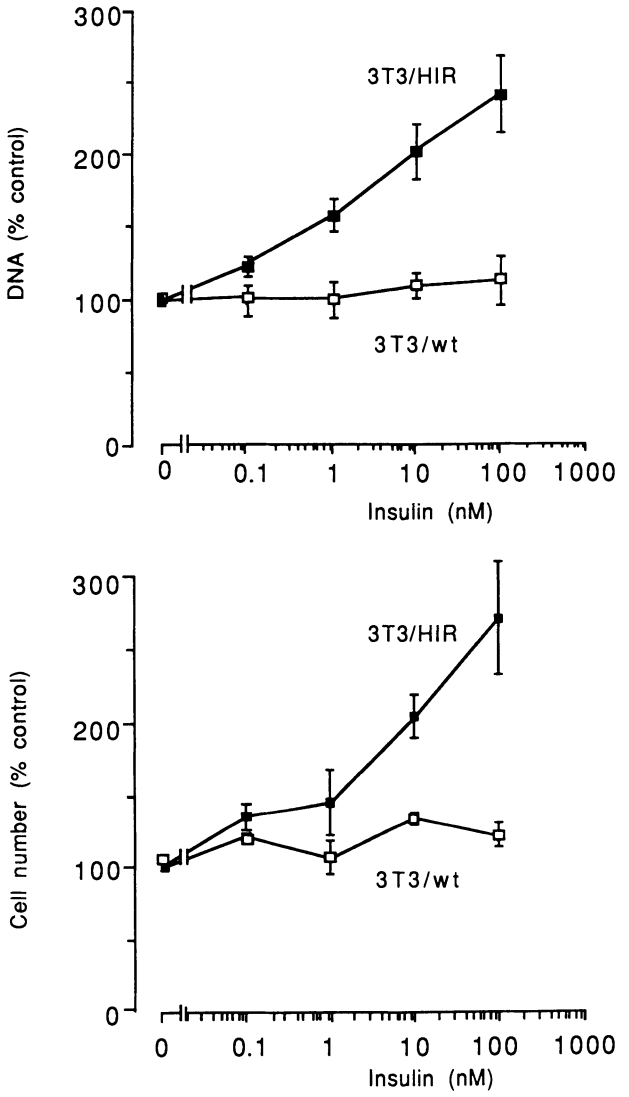

Figure 11. Proliferation of 3T3/wt and 3T3/HIR cells in response to insulin. Cells were cultured in the absence or presence of increasing concentrations of insulin for 4 days in serum-free medium containg 0.1% BSA. (From Giorgino et al. [25] with permission.)

the neomycin resistance gene (3T3/NEO), or the bovine papilloma virus (3T3/BPV).

To assess the presence of anchorage-independent growth, cells were seeded in soft agar and inspected for colony formation. 3T3/HIR cells showed absent or minimal colony growth in the absence of insulin. However, there was a dose-dependent, insulin-stimulated (Fig. 13) increase in both colony size and number (Table 2). Insulin-stimulated colony formation

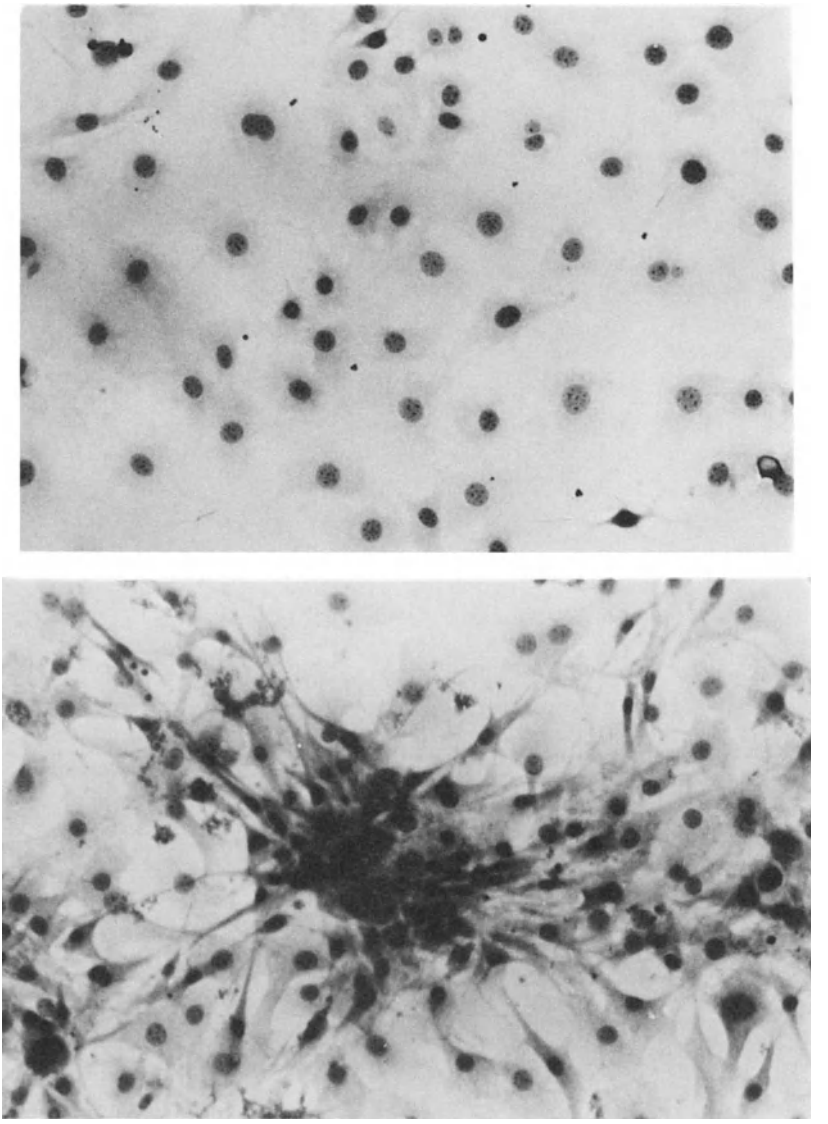

Figure 12. Light microscopic pictures of 3T3/HIR cells grown in medium containing 0.1% BSA in the absence (A) and presence (B) of 100 nM insulin. Cells were stained with Papanicolau stain. Magnification ×100. (From Giorgino et al. [25] with permission.)

was specifically inhibited by an insulin antagonist, the anti-insulin receptor monoclonal antibody MA-10. In the presence of 100 nM insulin, about 3% of cells formed large colonies. Insulin neither stimulated growth nor induced colony formation in 3T3/wt cells or 3T3/NEO cells. Insulin also stimulated

Table 2. Comparision of 3T3/HIR cell colony formation in soft agar in response to IGF-I and insulin

	Colonies	
Addition	Number	Size
None	85	Small
IGF-1 (nM)		
0.1	83	Small/medium
1.0	112	Small/medium
10.0	115	Small/medium
Insulin (nM)		
10.0	480	Large

From Giorgino et al. [25] with permission.

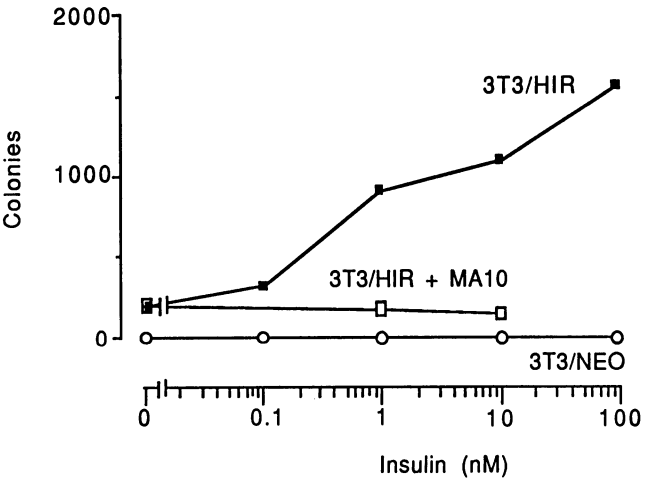

Figure 13. 3T3/HIR and 3T3/NEO cell soft agar colony formation in response to insulin. Cells were seeded in 0.33% soft agar in the absence or presence of increasing concentrations of insulin. (From Giorgino et al. [25] with permission.)

colony formation in CHO cells transfected with an insulin receptor cDNA construct. However, when 10^5 to 10^6 3T3/HIR or 3T3/NEO cells were injected in nude mice, no specific tumor formation was observed after 8 weeks. In conclusion, overexpression of normal insulin receptors induces a ligand-dependent transformed phenotype. This phenomenon may have clinical relevance by conferring a selective growth advantage to tumor cells with high numbers of insulin receptors and playing a role, therefore, in the initiation or progression of certain breast tumors.

Conclusions

The mechanism(s) whereby insulin receptors are increased in human breast cancer remain speculative. There are several possibilities. Since the genes for some oncogenes, including c-*myc*, *int*-2, and c-*erb*B-2 (HER-2/*neu*), may be amplified in human breast carcinomas [26], it is possible that the insulin receptor gene is also amplified in some breast cancers. Alternatively, since structural gene rearrangement occurs in human breast carcinomas [27], it is possible that the insulin receptor gene is altered in this tumor and consequently insulin receptor mRNA is overexpressed.

Our studies in three cultured breast cancer cell lines (two of which had increased insulin receptor content) indicated that neither the insulin receptor gene copy number nor the insulin receptor mRNA content were increased. Therefore it is possible that the processes of either enhanced protein synthesis or decreased protein degradation are involved in the mechanism of increased insulin-receptor content. It is also possible that either the insulin receptor signal transduction pathway or additional cell processes are altered in breast cancer cells, and that other hormones (such as progestins) play a role in regulating the insulin receptor content of breast cancers.

Insulin receptors in breast cancers were functional. They bind insulin and induce ligand-activated tyrosine kinase activity. Since insulin is a mitogen for a variety of cells, it is possible that insulin via the insulin receptor plays a role in breast cancer growth. Our studies with 3T3 and CHO cells indicate that when these cells express an increased number of insulin receptors they acquire a transformed phenotype, which is insulin dependent and is mediated via the insulin receptor. However, in these cells the expression of the insulin receptor does not induce tumor formation in nude mice. This observation suggests that, in addition to the insulin receptor, other factors may be necessary for full neoplastic transformation.

The appearance of an insulin-dependent transformed phenotype in 3T3 cells transfected with the insulin receptor gene resembles a similiar phenomenon described in NIH-3T3 cells transfected with the human epidermal growth factor (EGF) receptor gene [28–30]. 3T3 cells overexpressing EGF receptors demonstrated anchorage-independent growth when stimulated with either EGF or TGF-α. These observations suggest, therefore, that overexpression of insulin receptors and related receptors in the tyrosine kinase family may lead to the initiation and/or progression of certain tumors.

The clinical significance of the observation that increased insulin receptors are present in most breast cancers is unknown. Insulin receptor content correlates with other clinical parameters, including tumor size and grade, properties that reflect increased tumor aggressiveness. Tumor size reflects both tumor growth rate and the duration of tumor growth. These observations suggest, therefore, that less differentiated and more aggressive breast tumors contain more insulin receptors. It is also possible that the

duration of tumor growth leads to the selection of cancer cells with a higher insulin receptor content. On the other hand, there is no apparent correlation between insulin receptor content and lymph node involvement, an important reflection of breast tumor aggressiveness. Also, insulin receptor content appears to correlate with estrogen receptor content, a more favorable prognostic indicator. Thus, whether breast tumor insulin receptor content predicts for a negative or positive patient outcome remains to be determined, and these studies are now in progress.

Acknowledgments

The studies in this review were carried out in collaboration with A. Belfiore, A. Costantino, L. Frittitta, F. Giorgino, B. Maddux, G. Milazzo, V. Papa, V. Pezzino, V. Trischitta, and K.Y. Wong. These studies were supported in part by the John A. Kerner Foundation, the Jay Gershow Cancer Fund, and the Ladies Auxiliary of the Veterans of the Foreign Wars, and Associazione Italiana per la Ricerca sul Cancro.

References

1. Henderson IC, Harris JR, Kinne DW, Hellman S: In: DeVita VT Jr., Hellman S, Rosenberg SA (eds): Cancer, Principles and Practice of Oncology, 3rd Ed. JB Lippincott, Philadelphia, 1989, pp ●●–●●.
2. Silverberg E, Lubera JA: Cancer 39:3, 1989.
3. Dickson RB, Lippman ME: Endocr Rev 8:29, 1987.
4. Clark GM, McGuire WL, Hubay WL: N Engl J Med 309:1343, 1983.
5. Wittliff JL: Cancer 53:630, 1984.
6. Maas H, Jonat W, Stolzenbach G, Trams G: Cancer 46:2835, 1980.
7. Yarden Y, Ullrich A: Ann Rev Biochem 57:443, 1988.
8. Lee PL, Johnson DE, Cousens LS, Fried VA, Williams LT: Science 245:57, 1989.
9. Hennipman A, van Orischot BA, Smits J, Rijksen G, Staal GEJ: Cancer Res 49:516, 1989.
10. Slamon DJ, Godolphin W, Jones LA, et al.: Science 244:707, 1989.
11. Tandon AK, Clark GM, Chamness GC, Ullrich A, McGuire WL: J Clin Oncol 7:1120, 1989.
12. Heuson, JC, Coune A, Heimann R: Exp Cell Res 45:351, 1967.
13. Welsh CW, Calaf De Iturri G, Brennan MJ: Cancer 38:1272, 1976.
14. Shafie SM, Hilf R: Cancer Res 38:759, 1978.
15. Van Der Burg B, Rutteman GR, Blankenstein MA, De Laat SW, Van Zoelen EJJ: J Cell Physiol 134:101, 1988.
16. Gross GE, Boldt DH, Osbrone CK: Cancer Res 44:3570, 1984.
17. Osborne CK, Bolan G, Monaco ME, Lippman ME: Proc Natl Acad Sci USA 73:4536, 1976.
18. Mountjoy KG, Finlay GJ, Holdaway IM: Cancer Res 47:6500, 1987.
19. Osborne CK, Monaco ME, Lippman ME, Kahn CR: Cancer Res 38:94, 1978.
20. Mountjoy KG, Holdaway IM, Finlay GJ: Cancer Res 43:4537, 1983.
21. Benson EA, Holdaway IM: Cancer Res 42:1137, 1982.
22. Pezzino V, Papa V, Trischitta V, et al.: Am J Physiol 257:E451, 1989.
23. Papa V, Pezzino V, Costantino A, et al.: J Clin Invest 86:1503, 1990.

24. Papa V, Reese CC, Brunetti A, et al.: Cancer Res 50:7858, 1990.
25. Giorgino F, Belfiore A, Milazzo G, et al.: Mol Endocrinol 5(3):452, 1991.
26. Ali IU, Campbell G, Lidereau R, Callahan R: Science 240:1795, 1988.
27. T'Ang A, Varley JM, Chakraborty S, Murphree AL, Fung YKT: Science 242:263, 1988.
28. Di Fiore PP, Pierce JH, Fleming TP, et al.: Cell 51:1063, 1987.
29. Velu TJ, Beguinot L, Vass WC, et al.: Science 238:1408, 1987.
30. Riedel H, Massoglia S, Schlessinger J, Ullrich A: Proc Natl Acad Sci USA 85:1477, 1988.

10. The role of fibroblast growth factors and related oncogenes in tumor growth

Harold Brem and Michael Klagsbrun

The FGF family

Introduction

The fibroblast growth factors (FGF) constitute a family of seven mitogenic and structurally homologous polypeptides found in a variety of cells and tissues [for reviews see 1–9]. The FGF family includes acidic FGF (aFGF), basic FGF (bFGF), *int*-2, *hst*/K-*fgf*, FGF-5, FGF-6, and keratinocyte growth factor (KFGF) (Table 1). A simplified nomenclature has been proposed in which the FGF family members are named FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6, and FGF-7, respectively. Structurally, the homologies between the seven FGF family members is 35–45%, with the homologies being greatest in the internal regions of these proteins. Their molecular weights range from 18 to 30 kDa. They all share with aFGF and bFGF the 3 exon-2 intron structure and the conservation of two cysteine residues. An important structural difference between the FGF family members is that, unlike the others, aFGF and bFGF lack signal peptide sequences and are not secreted proteins. Members of the FGF family, in particular bFGF [10], are also characterized by their strong affinity for heparin. The affinity of bFGF for heparin is manifested in its ability to bind to cell surface heparan sulfate proteoglycan (HSPG), an activity that is required for binding to high-affinity FGF receptors [9]. A schematic representation of the FGF family members portraying domains of sequence homology and signal peptides is shown in Fig. 1.

The biological significance of cell-associated aFGF and bFGF is still a matter of conjecture, while the other five FGFs are thought to be involved in typical paracrine and autocrine growth mechanisms. An important biological distinction is that aFGF, bFGF, and KGF do not transform the cells that produce them, while *int*-2, *hst*/K-*fgf*, FGF-5, and FGF-6 are oncogenes. Unlike aFGF and bFGF, which are found ubiquitously in adult tissue, these oncogenes are expressed primarily during embryogenesis, neonatal development, and in many tumors. KGF is associated mostly with the epithelium.

In this chapter, we will review the structural and biological properties of

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Table 1. Members of the FGF family

	Common name	MW	Originally found in
FGF-1	acidic FGF (HBGF-1)	18,000	Adult tissue (neural)
FGF-2	basic FGF (HBGF-2)	18,000	Most adult tissue
FGF-3	*int*-2	27,000	Site of MMTV integration, breast carcinoma
FGF-4	*hst*/K-*fgf*	23,000	Human stomach tumor (*hst*), Kaposi's sarcoma (K*FGF*)
FGF-5	*FGF* 5	29,000	Bladder carcinoma, hepatoma
FGF-6	FGF 6	?	Homologous to *hst*
FGF-7	Keratinocyte Growth factor (KGF)	28,000	Epithelial tissue stromal cells

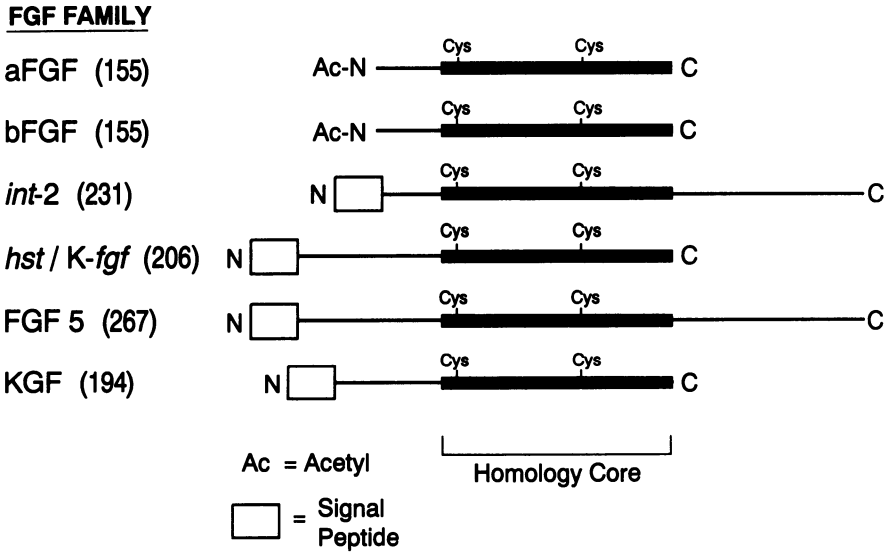

Figure 1. Schematic representation depicting FGF family structural features. The numbers in parentheses represent the numbers of amino acids in the open reading frames. Also depicted are the presence or absence of signal peptide sequences, the N-terminal amino acids blocked by acetylation, the regions of structural homology, and the presence throughout the family of two homologous cys residues.

the individual FGF family members, with an emphasis on their perceived role in tumorigenesis.

aFGF and bFGF (FGF-1 and FGF-2)

Acidic FGF and basic FGF will be discussed together, since only subtle physiological differences have been found between these two well-charac-

terized proteins. Acidic FGF and bFGF have a 53% protein sequence homology [11]. Both are single-chain polypeptides of 154 amino acids with molecular weights of about 18 kDa, as predicted from their cDNA nucleotide sequence. The gene for aFGF is located on chromosome 5 [12], while that of bFGF is located on chromosome 4 [13]. They also have other important structural differences [6,13–15]. Acidic FGF is an anionic protein with a pI of 5.6 [15], while bFGF is very cationic with a pI of about 10 [16]. In addition, there are forms of bFGF containing more than 154 amino acids that have molecular weights of 22–25 kDa [17–19]. These higher molecular weight forms of bFGF are generated by an unusual mechanism in which synthesis is initiated on the CUG start codons, rather than the typical AUG codon that initiates the 18 kDa form [18,19].

An important property of aFGF and bFGF is their interaction with heparin [20,21]. Both bind tightly to columns of immobilized heparin, a property that has facilitated their purification [1,10,15,20]. In addition, heparin stabilizes aFGF and bFGF, and protects them from heat, acid [21], and proteolytic degradation [22]. Acidic FGF and bFGF also bind to heparin-like molecules that are associated with cells. These include heparan sulfate proteoglycans (HSPG) in extracellular matrix and on cell surfaces. It has been suggested that aFGF and bFGF are sequestered or "stored" in the extracellular matrix [23–25] as part of a highly stable FGF-HSPG complex and are released during injury by a combination of proteases and heparinases [23]. The binding of bFGF to cell surface HSPG is a prerequisite for FGF's ability to bind to the FGF high-affinity receptor [9,26] as well as for bFGF mitogenic activity. Thus, heparin binding is an important property that modulates FGF structure, stability, *and* function. Interestingly, bFGF also maintains a high affinity for betacyclodextrin-tetradecasulfate, which structurally resembles heparin [27].

The biological activities of aFGF and bFGF are very similar. Both are important components of endothelial cell growth and differentiation, and stimulate new blood vessel growth, i.e., they are angiogenic in vivo [1]. Their angiogenic activity stems from the ability to stimulate many components in the formation of new blood vessels, such as (1) endothelial cell migration [28], (2) endothelial cell proliferation [29], (3) protease production [30], (4) matrix degradation [31], (5) plasminogen activator activity [32], and (6) capillary tube formation [33].

Acidic FGF and bFGF also stimulate the proliferation of a variety of other cell types in vitro, including fibroblasts [34], vascular smooth muscle cells [35], granulosa cells [36], osteoblasts [37], ovarian epithelial cells [38], oligodendrocytes [39], and keratinocytes [40].

There has been considerable effort to analyze the activities of aFGF and bFGF in vivo. One fruitful area has been the role of these growth factors in modulating wound healing. Since endothelial cells and fibroblasts are critical components of wound healing, mitogens of these cell types, such as aFGF and bFGF, might be expected to accelerate wound repair. In one of the first

studies to elucidate the role of fibroblast growth factor in wound healing, it was demonstrated that bFGF administered to a wound stimulates the formation of a highly vascular granulation tissue [41]. Subsequently, it was found that topical application of bFGF increased tensile strength in sutured linear incisions in rats [42] and accelerated the rate of closure of partial-thickness wounds in pigs [43]. If bFGF is blocked in vivo by local application of antibodies, the wound will not heal, suggesting that bFGF has a natural critical role in wound healing [44].

Folkman and colleagues hypothesized that duodenal ulcers are similar to surgical wounds that require bFGF to heal. Therefore they administered an acid-resistant oral form of bFGF to rats and found that angiogenesis was stimulated in the ulcer bed and the ulcers healed significantly more rapidly [45].

Acidic FGF is also active in vivo. When applied in Gelfoam implants in the peritoneal cavities of rats [46], aFGF stimulates angiogenesis.

One potential important therapeutic use for aFGF, bFGF, and KFGF in patients may be to reverse impaired wound healing. In rodents, the application of topical bFGF has been demonstrated to reverse wound healing impairments produced by systemic steroids and genetic obesity [47], genetically induced diabetes [48], and local bacterial contamination [49]. These results may be applicable to humans as well.

Fibroblast growth factor may also be useful in stimulating a variety of regenerative processes in the central nervous system. For example, bFGF increases neuronal preservation [50] and nerve regeneration [51].

int-*2 (FGF-3)*

int-2 was the first FGF-like oncogene to be described [52–55]. It was so named because of the initial discovery that it was induced to become transcriptionally active after integration (*int*) of the mouse mammary tumor virus into the mouse genome [53]. The *int*-2 gene is expressed in very specific time periods and locations from midgestation until birth in amphibia [56–58]. The *int*-2 gene induces mesoderm in *Xenopus laevis* animal pole cells and stimulates DNA synthesis in mammalian fibroblasts [59]. Based on these studies and others, it has been designated a developmental control gene [56].

Once the structure of *int*-2 was determined its homology to bFGF became apparent. The *int*-2 gene encodes for a protein of 231 amino acids that has a 46% homology to bFGF [60]. As expected for a secreted protein with a signal peptide, the *int*-2 protein can be detected in the endoplasmic reticulum of transfected cells [61]. However, the *int*-2 protein has not, to date, been shown to be an active mitogen and its mechanism in transforming cells is unclear.

As an oncogene induced by mammary tumor virus, *int*-2 would be expected to be expressed by breast tumors. Expression of *int*-2 in transgenic

mice results in epithelial cell hyperplasia in mammary and salivary glands, as well as prostatic epidermal hypertrophy, which results in male sterility [62]. Amplification of the *int*-2 gene has been found in a variety of human tumors, particularly in breast carcinomas [63–66] and squamous cell carcinomas of the head and neck region [66–69]. The clinical implications of these findings appear to be relatively insignificant, since they occur in less than a majority of patients with these tumors and have not been shown to have a significant correlation with outcome in patients in whom *int*-2 is expressed. In breast carcinoma, for example, the percentage of patients who show *int*-2 amplification is usually less than 30% [63–66].

Int-2 is expressed during embryogenesis and tumorigenesis; however, further work is needed to understand its function in oncogenically transforming cells. Since it is rarely found in adult preneoplastic cells, one possible function for *int*-2 is as a clinical prognosticator. Furthermore, its diagnostic significance may be amplified when it is found in the presence of other oncogenes [70–72]. The coamplification of oncogenes may be important for many oncogenes that have not yet had significant clinical impact when expressed in and of themselves. One exception is the expression of N-*myc* in neuroblastomas, which has clinical significance when it is singularly expressed [73].

hst/*K*-fgf *(FGF-4)*

The *hst*/K-*fgf* oncogene was isolated from two sources simultaneously. One source was NIH-3T3 cells transfected with the Kaposi sarcoma DNA, hence the name Kaposi FGF (K-fgf) [74,75]. The other source was NIH-3T3 cells transfected with DNA from a human stomach tumor, hence the name *hst* [76–78]. *hst*/K-*fgf* is located on chromosome 11 band q13 [77], approximately 40–50 kb from the *int*-2 gene. The *hst*/k-*fgf* has 43%, 38%, and 40% sequence homologies to aFGF, bFGF, and *int*-2, respectively.

hst/K-*fgf* has similar biological activities to aFGF and bFGF, but it has different structural features. In particular, the *hst*/K-*fgf* gene encodes for a 206 amino acid primary translation product that contains a hydrophobic signal peptide sequence. In distinction to aFGF and bFGF, the mature 23-kDa 176 amino acid protein of *hst*/k-*fgf* is glycosylated and secreted [75]. The gene for *hst*/K-*fgf* is rarely expressed in adult cells or in adult tissues [79]. It is expressed, however, in embryogenesis, specifically during mid-stage mouse embryogenesis. *hst*/k-*fgf* also stimulates DNA synthesis in mammalian fibroblasts [59]. As expected for an oncogene, *hst*/K-*fgf* synthesis has been demonstrated in a variety of solid tumors, including germ cell [80], esophageal [81], gastric [82], and breast tumors [83]. On the other hand, it is not expressed in hematopoietic tumors, such as leukemias [83]. Interestingly, although *hst*/K-*fgf* was first isolated from cells transfected with Kaposi sarcoma DNA, it has not been detected in the secreted material from cultured Kaposi sarcoma cells [84,85].

It has been suggested that *hst*/K-*fgf* induces the transformed phenotype by binding to cell surface receptors, thereby creating an autocrine closed loop [75]. No specific receptor has been identified for the *hst*/K-*fgf* protein, but it is thought to bind to the same receptor as aFGF and bFGF [85].

FGF-5

FGF-5 was originally isolated by transfection of a human bladder tumor DNA into NIH-3T3 cells [86]. Its gene sequence has 40–50% homology to aFGF and bFGF [87]. The FGF-5 gene is found on human chromosome number 4 [88] and encodes for a 267 amino acid protein with a signal sequence. There is some evidence that FGF-5 synthesis is dramatically increased if there is a deletion or a point mutation in an upstream open reading frame [89].

The FGF-5 protein is secreted as glycoprotein molecules of heterogeneous sizes [89]. It is a potent mitogen for endothelial cells and fibroblasts [87]. Messenger RNA transcripts for FGF-5 are found in nearly all phases of embryogenesis [90] and in the neurons of adult brains [91]. The protein for FGF-5 is also secreted from bladder carcinoma, endometrial carcinoma, and human hepatoma cell lines [87].

FGF-6

FGF-6 is an oncogene originally isolated from a mouse plasmid library by screening with the *hst*/K-*fgf* gene [92]. The FGF-6 gene is found on chromosome 12 band p13, unlike *int*-2 and *hst*/K-*fgf*, which are localized on chromosome 11 band q13 [93]. Transfection of NIH-3T3 cells with the FGF-6 gene transforms them. The amino acid sequence for FGF-6 is 70% identical to the aFGF product at the C terminus. FGF-6 is the least characterized of the FGF family members and there are very little data to date on expression of the FGF-6 protein.

KGF

The keratinocyte growth factor (KGF) has a 39% homology to bFGF [94]. The KGF gene encodes for a primary translation product of 194 amino acids, and the mature protein does have a signal peptide and is secreted. KGF is present in stromal cells (i.e., fibroblasts) derived from epithelial tissues and is present in embryonic and adult tissue [94]. It has not to date been identified in tumor cells. Unlike other FGF family members, it is neither an endothelial cell growth/angiogenesis factor nor is it an oncogene. Instead, it is a highly specific mitogen for epithelial cells, in general, and for keratinocytes in particular [94]. Thus it differs from aFGF and bFGF, which do not show such target cell specificity.

Table 2. Presence of fibroblast growth factors in human tumors

Tumor	aFGF	bFGF	*int*-2	*hst*/K-*fgf*	FGF-5	References
Adrenal carcinoma		+				175
Basal cell carcinoma		+				177
Bladder carcinoma		+			+	86,147
Brain tumors						
Gioblastoma	+	+				170–173
Meningioma		+				171
Acoustic neuroma		+				150
Pituitary tumors		+				151
Astrocytoma		+				174
Breast carcinoma		+	+	+		63–66,83, 152–155 169
Cervical carcinoma		+				160
Chollangiocellularcarcinoma		+				156
Colon carcinoma		+				78,179
Embryonal carcinoma		+		+		157,163
Endometrial carcinoma					+	87
Esophageal squamous cell carcinoma			+	+		67–69,81 72,158,159
Gastric adenocarcinoma				+		72,77,78,82
Hepatoma		+				87,160–162
Kaposi sarcoma		+				164,181
Laryngeal squamous cell carcinoma			+			67
Melanoma		+	+	+		70,160,177
Neuroblastoma	+	+				165,177
Oral cavity & tongue			+			67
Osteosarcoma		+				177
Ovarian carcinoma			+			166
Pancreatic adenocarcinoma		+				167
Renal cell carcinoma		+				147,149,168
Rhabdomyosarcoma	+	+				178,180
Teratocarcinoma		+	+	+		80,176
Tonsil squamous cell carcinoma			+			67

The FGF family and tumor growth

Members of the FGF family are expressed in animal and human tumors (for human tumors see, Table 2). The four FGF oncogenes, *int*-2, *hst*/K-*fgf*, FGF-5, and FGF-6 are involved in autocrine transformation of cells. The role of aFGF and bFGF is less clear, since they are found in both normal and tumor cells. Furthermore, tumorigenicity has not been directly correlated with aFGF or bFGF expression. Normal endothelial cells synthesize more bFGF than is expressed in many tumor cell lines [24,95]. The normal phenotype of these endothelial cells is maintained, even though these cells have FGF receptors and could in theory participate in autocrine transformation. Secondly, aFGF and bFGF are not secreted. Thus, even if expressed by tumor cells they may not be able to iduce autocrine trans-

formation, which typically requires interaction of a *secreted* growth factor with its receptor on the same cell type. Lack of active secretion also limits possible paracrine activity, unless aFGF or bFGF are released by alternative mechanisms, such as cell death.

There are conditions, however, in which aFGF or bFGF might induce autocrine cell transformation; for example, acquisition by FGF of a signal peptide. Cells transfected with native bFGF cDNA and overexpressing bFGF acquire an enhanced proliferation rate and a higher saturation density, evidence of a transformed phenotype. However, they remain density arrested and are nontumorigenic in syngeneic mice [96], suggesting that transformation in vitro is not necessarily correlated with tumorigenicity in vivo. Cells transfected with native aFGF cDNA are similarly nontumorigenic [97]. One possible explanation is that cells overexpressing the FGFs might release small but sufficient amounts of growth factor for stimulating autocrine growth in vitro but that in vivo this material diffuses away. Alternatively, aFGF and/or bFGF transform cells in culture by some type of internal autocrine mechanism in which FGFs are not released but interact with intracellular FGF receptors. This might occur in vitro to stimulate transformation but for some unknown reason may be insufficient to induce tumorigenicity in vivo.

Cells transfected with a construct in which bFGF cDNA is altered by addition of a signal sequence undergo autocrine transformation and exhibit morphological and biochemical alterations characteristic of highly transformed cells [96,98]. The signal peptide bFGF (spbFGF)-transformed cells have an accelerated proliferation rate, are not density arrested, and are capable of anchorage-independent growth. spbFGF cells possess few functional FGF receptors at the cell surface, supporting the idea that these cells are transformed by constitutive interaction with and downregulation of the FGF receptor. Most importantly, the spbFGF-transformed cells are highly tumorigenic and metastatic. It has been suggested that spbFGF transforms cells via an internal autocrine loop, since these cells do not secrete biologically active bFGF, despite the presence of a signal peptide, and their proliferation rate is not affected by neutralizing antibodies to bFGF.

The molecular mechanism by which a signal peptide-bearing bFGF leads to transformation is yet to be determined. It is possible that a structural, posttranslational modification of FGF, being processed through the endoplasmic reticulum and golgi apparatus, may lead to an atypical interaction with the FGF receptor. The localization of bFGF-receptor interaction might play an important role in autocrine transformation. While native bFGF can interact with the FGF receptor only at the cell surface, spbFGF might be able to bind the receptor inside the cell anywhere along the secretory pathway. Such an intracellular interaction may activate different modes of signal transduction by exposing novel substrates to the tyrosine kinase activity of the FGF receptor.

In summary, the four FGF oncogenes and their respective proteins are

the most likely to stimulate tumor growth. The reason for their oncogenic potential could be that, unlike aFGF and bFGF, these four oncogenes have naturally occurring signal sequences and encode for secreted proteins that appear to be involved in the autocrine transformation of cells possessing FGF receptors. In this regard, the distribution of *int*-2, *hst*/K-*fgf*, and FGF-5 is quite different than that of aFGF or bFGF. The oncogenes are rarely found in normal adult tissue. Rather, they appear to be mostly expressed during embryogenesis and in tumors. It is possible that FGF-related oncogenes are the forms of FGF preferentially expressed during periods of intense proliferation.

The FGF family and tumor vascularization

Proliferation of blood vessels is necessary for the normal growth and development of tissue. In the adult, angiogenesis occurs infrequently. Exceptions are found in the female reproductive system, where angiogenesis occurs in the follicle during its development, in the corpus luteum during ovulation, and in the placenta during pregnancy. These periods of angiogenesis are relatively brief and tightly regulated. Normal angiogenesis also occurs as part of the body's repair processes; for example, in the healing of wounds and fractures. By contrast, uncontrolled angiogenesis is usually pathological. For example, the ability of tumors to stimulate angiogenesis or new capillary blood vessel growth allows them to grow in an exponential manner [1,5,99–109]. The corollary of this principle is that without the ability of tumors to stimulate new blood vessels, the tumors will remain in a small, avascular state. Vascularization of a tumor also enhances metastatic potential. It has been recently demonstrated that in breast cancer patients their is a strong correlation between the number and density of microvessels in the primary tumor and the incidence of breast cancer metastases [110].

A number of growth factors have been shown to be angiogenic, including aFGF, bFGF, angiogenein, platelet-derived endothelial cell growth factor, vascular endothelial growth factor, tumor necrosis factor, and transformig growth factors-α and -β [1,4,7,111–114]. Acidic FGF and bFGF are the best characterized of all the angiogenesis factors. These FGFs stimulate angiogenesis in the classical bioassays, such as the normally avascular cornea and the chick chorioallantoic membrane [115, 116]. Acidic FGF and bFGF modulate endothelial cell activity in vitro in a manner consistent with being stimulators of angiogenesis in vivo. For example, in culture FGF stimulates endothelial cell chemotaxis [28] and proliferation [29] for endothelial cells. Endothelial cells themselves secrete substantial amounts of bFGF [29,95], most of which is associated with the subendothelial cell extracellular matrix [23–25,117–120]. FGF found in the endothelial cell extracellular matrix is an essential component required for blood vessel growth. It has been suggested that capillary growth is regulated locally by bFGF stored in

capillary basement membrane that is released to stimulate capillary endothelial cells in an autocrine manner [25,117]. Basic FGF has been shown to support tumor growth by stimulating blood vessel growth [121–123]. For example, colon carcinoma can be stimulated by the application of bFGF in vivo [121]. Since FGF receptors do not appear on the colon cancer cells themselves but are found on the endothelial cells in the tumor, it appears that colon carcinoma growth is mediated by bFGF-induced neovascularization. Neutralizing FGF antibodies significantly reduce the tumor volume, further suggesting an endothelial cell-stimulating paracrine role for FGF in the growth of some tumors [121,122]. Furthermore, when fibroblasts were transfected with a gene posessing a signal sequence fused to bFGF, large tumors grew in nude mice [123]. Antibodies to bFGF administered systemically resulted in approximately a 75% decrease in the size of these tumors [123].

An important question remains: How can bFGF, which is not normally secreted, become a paracrine vascularization factor? Several possible mechanisms of FGF release by tumors have been postulated, including tumor necrosis, tumor cell leakiness, acquisition of signal peptide, and induction of multidrug resistant genes that encode for proteins that are involved in FGF export. A possible mechanism involving differential bFGF export by normal and tumor cells has been reported [124]. In these studies, transgenic mice carrying the bovine papilloma virus genome (BPV-1) at first produced benign avascular dermal fibromatoses. Eventually, there was a transition from the avascular tumors to the formation of highly vascular malignant fibrosarcomas. The *switch* from avascular to vascular tumors was accompanied by a change in bFGF release profiles. Basic FGF was expressed in both normal dermal fibroblasts and in benign fibromatoses but was cell associated, a typical property of bFGF that has no signal peptide for secretion. In contrast, the fibrosarcoma cells had very little cell-associated bFGF and a substantial amount of exported bFGF-like activity, which was neutralized by anti-bFGF antibodies. Since bFGF is angiogenic, it may be that its export by fibrosarcoma cells results in a paracrine stimulation of blood vessel growth in the tumors. Tumor angiogenesis might not occur in the avascular fibromatoses because bFGF is not released by these tumor cells. The mechanism by which the fibrosarcoma cells export bFGF is not understood. The cells might have special pathways for exporting proteins, or alternatively, the bFGF in these cells might be altered structurally and exported. The precise nature of this mechanism is not understood to date.

Conclusions and future directions

Members of the FGF family are important modulators of tumor growth. The four FGF oncogenes — *int*-2, *hst*/K-*fgf*, FGF-5, and FGF-6 — are the most likely candidates to be involved in stimulating autocrine tumor growth and

tumor neovascularization because they are secreted. The role of aFGF and bFGF in tumor growth is less clear. Since they are not secreted proteins, it is not clearly understood how they could be involved in cell transformation. Recent studies have suggested some possible mechanisms that allow aFGF and bFGF to be involved in tumor growth. For example, internal autocrine loops may occur in which aFGF and bFGF interact with their receptors within cells. Another possible mechanism is the alteration of FGF structure or of the tumor cell that allows specific FGF export, resulting in the stimulation of tumor angiogenesis.

Given the possibility that FGF is involved in tumor growth, anti-bFGF therapy might have therapeutic value. Several strategies have been attempted. These include the following: (1) the use of neutralizing antibodies that would inhibit exported members of the FGF family [123], (2) the use of antisense oligonucleotides that inhibit FGF synthesis. Basic FGF antisense has been used to inhibit melanoma growth [125]. (3) The use of drugs that inhibit the interaction of FGF with its receptor. Suramin has been shown to inhibit FGF-FGF receptor interactions and to revert the phenotype of tumors dependent on FGF production [126,127]. (4) The use of reagents that degrade cell surface heparan sulfate proteoglycans (HSPG). It has been shown that bFGF needs to bind to cell surface HSPG in order to be mitogenic [9,26,128]. Inactivating cell surface HSPG with heparinase or with specific peptides that bind to HSPG might be a way to block the mitogenic activity of FGF family members in a tumor. (5) The use of angiogenesis inhibitors. These compounds include a synthetic laminin peptide [129], AGM 1470 [130–133]), minocycline [134], thrombospondin [135,136], cartilage-derived inhibitor [137], penicillamine [138,139], platelet-factor 4 [140], and modulators of collagen metabolism [141]. Angiostatic steroids with or without heparin [142,143] or with betacyclodextrin-tetradecasulfate [144] have also been found to be potent angiogenesis inhibitors. The extent to which these angiogenesis inhibitors interfere with FGF expression remains to be elucidated.

The presence of FGF family members in tissues and biological fluids [145–147] may have diagnostic value. Methods that could be used to detect these growth factors include immunocytochemistry of tumor tissue, in situ hybridization to detect FGF family transcripts, and ELIZA analysis [148,149].

There is a great deal still to be learned about the FGF family, including questions of structure, localization, biosynthesis, regulatory mechanisms, and involvement in angiogenesis. More detailed information regarding these FGF properties might continue to provide information that can be translated into further advances in the diagnosis and therapy of benign and malignant diseases.

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References

1. Folkman J, Klagsbrun M: Angiogenic factors. Science 235:442–447, 1987.
2. Rifkin DB, Moscatelli D: Recent developments in the cell biology of basic fibroblast growth factor. J Cell Biol 109:1–6, 1989.
3. D'Amore PA: Modes of FGF release *in vivo* and *in vitro*. Cancer Met Rev 9:227–238, 1990.
4. Klagsbrun M, Folkman J: Angiogenesis. In: Sporn MB, Roberts AB (eds): Handbook of Experimental Pharmacology, Peptide Growth Factors and their Receptors, Part II, Vol. 95. Springer-Verlag, Berlin, 1990, pp 549–586.
5. Folkman J: The angiogenic activity of FGF and its possible clinical applications. In: Sara VR, Hall K, Low H (eds): Karolinska Institute Nobel Conference Series on Growth Factors, Growth Factors: From Genes to Clinical Application. Karolinska Institute Nobel Conference Series. Raven Press, New York, 1990, pp 201–216.
6. Klagsbrun M: The fibroblast growth factor family: Structural and biological properties. Progr Growth Factor Res 1:207–235, 1989.
7. Klagsbrun M, D'Amore PA: Regulators of angiogenesis. Annu Rev Physiol 53:217–239, 1991.
8. Klagsbrun M: The affinity of fibroblast growth factors (FGF's) for heparin; FGF-heparan sulfate interactions in cells and extracellular matrix. Curr Opin Cell Biol 2:857–863, 1990.
9. Klagsbrun M, Baird A: A dual receptor system is required for basic fibroblast growth factor activity. Cell 67:229–231, 1991.
10. Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M: Heparin affinity: Purification of a tumor-derived capillary endothelial cell growth factor. Science 223:1296–1299, 1984.
11. Esch F, Baird A, Ling N, Ueno N, Hill F, Denoroy L, Klepper R, Gospodarowicz D, Bohlen P, Guillemin R: Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine brain acidic FGF. Proc Natl Acad Sci USA 82:6507–6511, 1985.
12. Jaye M, Howk R, Burgess W, Ricca GA, Chiu I-M, Ravera MW, O'Brien SJ, Modi WS, Maciag T, Drohan WN: Human endothelial cell growth factor: Cloning, nucleotide sequence, and chromosome localization. Science 233:541–545, 1986.
13. Abraham JA, Mergia A, Whang JL, Tumolo A, Friedman J, Hjerrild KA, Gospodarowicz D, Fiddes JC: Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. Science 233:545, 1986.
14. Thomas KA, Rios-Candelore M, Fitzpatrick S: Purification and characterization of acidic fibroblast growth factor from bovine brain. Proc Natl Acad Sci USA 81:357–361, 1984.
15. Burgess W, Maciag T: The heparin-binding (fibroblast) growth factor family of proteins. Ann Rev Biochem 58:575–606, 1989.

16. Gospodarowicz D: Fibroblast growth factor. In: Pimental E, Perucho M (eds): Critical Reviews in Oncogenesis. CRC Press: Boca Raton, Fl, 1989, pp 1–26.
17. Moscatelli D, Joseph-Silverstein J, Manejias R, Rifkin DB: M_r 25,000 heparin-binding protein from guinea pig brain is a high molecular weight form of basic fibroblast growth factor. Proc Natl Acad Sci USA 84:5778–5782, 1987.
18. Prats H, Kaghad M, Prats AC, Klagsbrun M, Lelias JM, Liauzun P, Chalon P, Tauber JP, Amalric F, Smith JA, Caput D: High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. Proc Natl Acad Sci USA 86:1836–1840, 1989.
19. Florkiewicz RZ, Sommer A: Human basic fibroblast growth factor gene encodes four polypeptides: Three initiate translation from non-AUG codons. Proc Natl Acad Sci USA 86:3978–3981, 1989.
20. Klagsbrun M, Shing Y: Heparin affinity of anionic and cationic capillary endothelial cell growth factors: Analysis of hypothalamus-derived growth factors and fibroblast growth factors. Proc Natl Acad Sci USA 82:805–809, 1985.
21. Gospodarowicz D, Cheng J: Heparin protects basic and acidic FGF from inactivation. J Cell Physiol 128:475–484, 1986.
22. Sommer A, Rifkin DB: Interaction of heparin with human basic fibroblast growth factor: Protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. J Cell Physiol 138:215–220, 1989.
23. Bashkin P, Doctrow S, Klagsbrun M, Svahn CM, Folkman J, Vlodavsky I: Basic fibroblast growth factor binds to subendothelial extracellular matrix and is released by heparitinase and heparin-like molecules. Biochemistry 28:1737–1743, 1989.
24. Vlodavsky I, Folkman J, Sullivan R, Fridman R, Ishai-Michaeli R, Sasse J, Klagsbrun M: Endothelial cell-derived basic fibroblast growth factor: Synthesis and deposition into subendothelial extracellular matrix. Proc Natl Acad Sci USA 84:2292–2296, 1987.
25. Folkman J, Klagsbrun M, Sasse J, Wadzinski M, Ingber D, Vlodavsky I: A heparin-binding and angiogenic protein — basic fibroblast growth factor — is stored within basement membrane. Am J Pathol 130:393–400, 1988.
26. Yayon A, Klagsbrun M, Esko JD, Leder P, Ornitz DM: Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 64:841–848, 1991.
27. Shing Y, Folkman J, Weisz PB, Joullie MM, Ewing WR: Affinity of fibroblast growth factors for beta-cyclodextrin tetradecasulfate. Anal Biochem 185:108–111, 1990.
28. Stokes CL, Rupnick MA, Williams SK, Lauffenburger DA: Chemotaxis of human microvessel endothelial cells in response to acidic fibroblast growth factor. Lab Invest 63:657–668, 1990.
29. Schweigerer L, Neufeld G, Friedman J, Abraham JA, Fiddes JC, Gospodarowicz D: Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. Nature 325:257–259, 1987.
30. Gross JL, Moscatelli D, Rifkin DB: Increased capillary endothelial cell protease activity in response to angiogenic stimuli *in vitro*. Proc Natl Acad Sci USA 80:2623–2627, 1983.
31. Mignatti P, Tsuboi R, Robbins E, Rifkin DB: *In vitro* angiogenesis on the human amniotic membrane: Requirement for basic fibroblast growth factor-induced proteinases. J Cell Biol 108:671–682, 1989.
32. Presta M, Maier JA, Rusnati M, Moscatelli D, Ragnotti G: Modulation of plasminogen activator activity in human endometrial adenocarcinoma cells by basic fibroblast growth factor and transforming growth factor beta. Cancer Res 48:6383–6389, 1988.
33. Montesano R, Vassalli J-D, Baird A, Guillemin R, Orci L: Basic fibroblast growth factor induces angiogenesis in vitro. Proc Natl Acad Sci USA 83:7297–7301, 1986.
34. Gospodarowicz D: Localization of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. Nature 249:123–129, 1974.
35. Weich HA, Iberg N, Klagsbrun M, Folkman J: Expression of acidic and basic fibroblast

growth factors in human and bovine vascular smooth muscle cells. Growth Factors 2:313–320, 1990.
36. Gospodarowicz D, Ill CR, Birdwell CR: Effects of fibroblast and epidermal growth factors on ovarian cell proliferation *in vitro*. I. Characterization of the response of granulosa cells to FGF and EGF. Endocrinology 100:1108–1120, 1977.
37. Rodan SB, Wesolowski G, Thomas K, Rodan GA: Growth stimulation of rat calvaria osteoblastic cells by acidic fibroblast growth factor. Endocrinology 121:1917–1923, 1987.
38. Gospodarowicz D, Plouet J, Fujii DK: Ovarian germinal epithelial cells respond to basic fibroblast growth factor and express its gene: Implications for early folliculogenesis. Int J Dev Neuro 7:401–409, 1989. Endocrinology 125:1266–1276, 1989.
39. Besnard F, Perraud F, Sensenbrenner M, Labourdette G: Effects of acidic and basic fibroblast growth factors on proliferation and maturation of cultured rat oligodendrocytes.
40. Shipley GD, Keeble WW, Hendrickson JE, Coffey RJ Jr., Pittelkow MR: Growth of normal human keratinocytes and fibroblasts in serum-free medium is stimulated by acidic and basic fibroblast growth factor. J Cell Physiol 138:511–518, 1989.
41. Davidson JM, Klagsbrun M, Hill KE, Buckley A, Sullivan R, Brewer PS, Woodward SC: Accelerated wound repair, cell proliferation, and collagen accumulation are produced by a cartilage-derived growth factor. J Cell Biol 100:1219–1227, 1985.
42. McGee GS, Davidson JM, Buckley A, Sommer A, Woodward SC, Aquino AM, Barbour R, Demetriou AA: Recombinant basic fibroblast growth factor accelerates wound healing. J Surg Res 45:145–153, 1988.
43. Hebda PA, Klingbeil CK, Abraham JA, Fiddes JC: Basic fibroblast growth factor stimulation of epidermal wound healing in pigs. J Invest Dermatol 95:626–631, 1990.
44. Broadley KN, Aquino AM, Woodward SC, Buckley-Sturrock A, Sato Y, Rifkin DB, Davidson JM: Monospecific antibodies implicate basic fibroblast growth factor in normal wound repair. Lab Invest 61:571–575, 1989.
45. Folkman J, Szabo S, Stovroff M, McNeil P, Li W, Shing Y: Duodenal ulcer: Discovery of a new mechanism and development of angiogenic therapy that accelerates healing. Ann Surg 214:414–427, 1991.
46. Thompson JA, Haudenschild C, Anderson KD, DiPietro JM, Anderson WF, Maciag T: Heparin-binding growth factor 1 induces the formation of organoid neovascular structures *in vivo*. Proc Natl Acad Sci USA 86:7928–7923, 1989.
47. Klingbeil CK, Cesar LB, Fiddes JC: Basic fibroblast growth factor accelerates tissue repair in models of impaired wound healing. In Barbul A, Caldwell MD, Eaglstein WH, Hunt TK, Marshall D, Pines E, Skover G (eds): Clinical and Experimental Approaches to Dermal and Epidermal Repair: Normal and Chronic Wounds. Wiley-Liss, New York, 1991, pp 443–458.
48. Greehalgh DG, Sprugel KH, Murray MJ, Ross R: PDGF and FGF stimulate wound healing in the genetically diabetic mouse. Am J Pathol 136:1235–1246, 1990.
49. Steinberg BD, Phillips LG, Hokanson JA, et al.: Effect of bFGF on the inhibition of contraction caused by bacteria. J Surg Res 50:47–50, 1991.
50. Abe K, Takayanagi M, Saito H: Effects of recombinant human basic fibroblast growth factor and its modified protein CS23 on survival of primary cultured neurons from various regions of fetal rat brain. Jpn J Pharmacol 53:221–227, 1990.
51. Otto D, Frotscher M, Unsicker K: Basic fibroblast growth factor and nerve growth factor administered in gel foam rescue medial septal neurons after fimbria fornix transection. J Neurosci Res 22:83–91, 1989.
52. Peters G, Brookes S, Smith R, Placzek M, Dickson C: The mouse homolog of the hst/k-FGF gene is adjacent to int-2 and is activated by proviral insertion in some virally induced mammary tumors. Proc Natl Acad Sci USA 86:5678–5682, 1989.
53. Dickson C, Smith R, Brookes S, Peters G: Tumorigenesis by mouse mammary tumor virus: Proviral activation of a cellular gene in the common integration region *int*-2. Cell 37:529–536, 1984.
54. Dickson C, Acland P, Smith R, Dixon M, Deed R, MacAllan D, Walther W, Fuller-Pace

F, Kiefer P, Peters G: Characterization of *int*-2: A member of the fibroblast growth factor family. J Cell Sci 13(Suppl.):87–96, 1990.
55. Muller WJ, Lee FS, Dickson C, Peters G, Pattengale P, Leder P: The *int*-2 gene product acts as an epithelial growth factor in transgenic mice. EMBO J 9:907–913, 1990.
56. Jakobovits A, Shackleford GM, Varmus HE, Martin GR: Two proto-oncogenes implicated in mammary carcinogenesis, *int*-1 and *int*-2, are independently regulated during mouse development. Proc Natl Acad Sci USA 83:7806–7810, 1986.
57. Wilkinson DG, Peters G, Dickson C, McMahon AP: Expression of the FGF-related proto-oncogene *int*-2 during gastrulation and neurulation in the mouse. EMBO J 7:691–695, 1986.
58. Wilkinson DG, Bhatt S, McMahon AP: Expression pattern of the FGF-related proto-oncogene *int*-2 suggests multiple roles in fetal development. Development 105:131–136, 1989.
59. Paterno GD, Gillespie LL, Dixon MS, Slack JM, Heath JK: Mesoderm-inducing properties of INT-2 and kFGF: Two oncogene-encoded growth factors related to FGF. Development 106:79–83, 1989.
60. Dickson C, Peters G: Potential oncogene product related to growth factors. Nature 326:833, 1987.
61. Dixon M, Deed R, Acland P, Moore R, Whyte A, Peters G, Dickson C: Detection and characterization of the fibroblast growth factor-related oncoprotein INT-2. Mol Cell Biol 9:4896–4902, 1989.
62. Ornitz DM, Moreadith RW, Leder P: Binary system for regulating transgene expression in mice: Targeting *int*-2 gene expression with yeast *GAL4/UAS* control elements. Proc Natl Acad Sci USA 88:698–702, 1991.
63. Tsuda H, Hirohashi S, Shimosato Y, Hirota T, Tsugane S, Yamamoto H, Miyajima N, Toyoshima K, Yamamoto T, Yokota J, Yoshida T, Sakamoto H, Terada M, Sugimura T: Correlation between long-term survival in breast cancer patients and amplification of two putative oncogene-coamplification units: *hst*-1/*int*-2 and c-*erb*B-2/*ear*-1. Cancer Res 49:3104–3108, 1989.
64. Meyers SL, O'Brien MT, Smith T, Dudley JP: Analysis of the *int*-1, *int*-2, c-*myc*, and *neu* oncogenes in human breast carcinomas. Cancer Res 50:5911–5918, 1990.
65. Machotka SV, Garrett CT, Schwartz AM, Callahan R: Amplification of the proto-oncogenes *int*-2, c-*erb* B-2 and c-*myc* in human breast cancer. Clin Chim Acta 184:207–218, 1989.
66. Zhou DJ, Casey G, Cline MJ: Amplification of human *int*-2 in breast cancers and squamous carcinomas. Oncogene 2:279–282, 1988.
67. Merritt WD, Weissler MC, Turk BF, Gilmer TM: Oncogene amplification in squamous cell carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg 116:1394–1398, 1990.
68. Kitagawa Y, Ueda M, Ando N, Shinozawa Y, Shimizu N, Abe O: Significance of *int*-2/*hst*-1 coamplification as a prognostic factor in patients with esophageal squamous carcinoma. Cancer Res 51:1504–1508, 1991.
69. Somers KD, Cartwright SL, Schechter GL: Amplification of the *int*-2 gene in human head and neck squamous cell carcinomas. Oncogene 5:915–920, 1990.
70. Adelaide J, Mattei M-G, Marics I, Raybaud F, Planche J, De Lapeyriere O, Birnbaum D: Chromosomal localization of the *hst* oncogene and its co-amplification with the *int*-2 oncogene in a human melanoma. Oncogene 2:413–416, 1988.
71. Ranzani GN, Pellegata NS, Previdere C, Saragoni A, Vio A, Maltoni M, Amadori D: Heterogeneous protooncogene amplification correlates with tumor progression and presence of metastases in gastric cancer patients. Cancer Res 50:7811–7814, 1990.
72. Tahara E: Growth factors and oncogenes in human gastrointestinal carcinomas. J Cancer Res Clin Oncol 116:121–131, 1990.
73. Hiyama E, Hiyama K, Yokoyama T, Ishii T: Immunohistochemical analysis of N-myc protein expression in neuroblastoma: Correlation with prognosis of patients. J Pediat Surg 26:838–843, 1991.

74. Delli-Bovi P, Curatola AM, Kern FG, Greco A, Ittman M, Basilico C: An oncogene isolated by transfection of Kaposi's sarcoma DNA encodes a growth factor that is a member of the FGF family. Cell 50:729–737, 1987.
75. Delli-Bovi P, Curatola AM, Newman KM, Sato Y, Moscatelli D, Hewick RM, Rifkin DB, Basilico C: Processing, secretion, and biological properties of a novel growth factor of the fibroblast growth family with oncogenic potential. Mol Cell Biol 8:2933–2941, 1988.
76. Yoshida T, Miyagawa K, Odagiri H, Sakamoto H, Little PFR, Terada M, Sugimura T: Genomic sequence of *hst*, a transforming gene encoding a protein homologous to fibroblast growth factors and the *int*-2-encoded protein. Proc Natl Acad Sci USA 84:7305–7309, 1987.
77. Yoshida MC, Wada M, Satoh H, Yoshida T, Sakamoto H, Miyagawa K, Yokota J, Koda T, Kakinuma M, Sugimura T, Terada M: Human *HST1* (*HSTF1*) gene maps to chromosome band 11q13 and coamplifies with the *INT2* gene in human cancer. Proc Natl Acad Sci USA 85:4861–4864, 1988.
78. Koda T, Sasaki A, Matsushima S, Kakinuma M: A transforming gene, *hst*, found in NIH 3T3 cells transformed with DNA from three stomach cancers and a colon cancer. Jpn J Cancer Res 78:325–328, 1987.
79. Delli-Bovi P, Mansukhani A, Ziff EB, Basilico C: Expression of the K-fgf protooncogene is repressed during differentiation of F9 cells. Oncogene Res 5:31–37, 1989.
80. Strohmeyer T, Peter S, Hartmann M, Munemitsu S, Ackermann R, Ullrich A, Slamon DJ: Expression of the *hst*-1 and c-*kit* protooncogenes in human testicular germ cell tumors. Cancer Res 51:1811–1816, 1991.
81. Tsuda T, Nakatani H, Matsumura T, Yoshida K, Tahara E, Nishihira T, Sakamoto H, Yoshida T, Terada M, Sugimura T: Amplification of the *hst*-1 gene in human esophageal carcinomas. Jpn J Cancer Res 79:584–588, 1988.
82. Sakamoto H, Mori M, Taira M, Yoshida T, Matsukawa S, Shimizu K, Sekiguchi M, Terada M, Sugimura T: Transforming gene from human stomach cancers and a noncancerous portion of stomach mucosa. Proc Natl Acad Sci USA 83:3997–4001, 1986.
83. Theillet C, Le Roy X, De Lapeyriere O, Grosgeorges J, Adnane J, Raynaud SD, Simony-Lafontaine J, Goldfarb M, Escot C, Birnbaum D, Gaudray P: Amplification of FGF-related genes in human tumors: Possible involvement of *HST* in breast carcinomas. Oncogene 4:915–922, 1989.
84. Ensoli B, Nakamura S, Salahuddin SZ, Biberfeld P, Larsson L, Beaver B, Wong-Staal F, Gallo RC: AIDS-Kaposi's sarcoma-derived cells express cytokines with autocrine and paracrine growth effects. Science 243:223–226, 1989.
85. Basilico C, Newman KM, Curatola AM, Talarico D, Mansukhani A, Velcich A, Delli-Bovi P: Expression and activation of the K-*fgf* oncogene. Ann NY Acad Sci 567:95–103, 1989.
86. Zhan X, Culpepper A, Reddy M, Loveless J, Goldfarb M: Human oncogenes detected by a defined medium culture assay. Oncogene 1:369–376, 1987.
87. Zhan X, Bates B, Hu X, Goldfarb M: The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. Mol Cell Biol 8:3487–3495, 1988.
88. Dionne CA, Kaplan R, Seuranez H, O'Brien SJ, Jaye M: Chromosome assignment by polymerase chain reaction techniques: Assignment of the oncogene FGF-5 to human chromosome 4. Biotechniques 8:190–194, 1990.
89. Bates B, Hardin J, Zhan X, Drickamer K, Goldfarb M: Biosynthesis of human fibroblast growth factor-5. Mol Cell Biol 11:1840–1845, 1991.
90. Hébert JM, Basilico C, Goldfarb M, Haub O, Martin GR: Isolation of cDNAs encoding four mouse FGF family members and characterization of their expression during embryogenesis. Dev Biol 138:454–463, 1990.
91. Haub O, Drucker B, Goldfarb M: Expression of the murine fibroblast growth factor 5 gene in the adult central nervous system. Proc Natl Acad Sci USA 87:8022–8026, 1990.
92. Marics I, Adelaide J, Raybaud F, Mattei M-G, Coulier F, Planche J, de Lapeyreire O,

Birnbaum D: Characterization of the HST-related FGF-6 gene, a new member of the fibroblast growth gene family. Oncogene 4:335–340.
93. de Lapeyriere O, Rosnet O, Benharroch, Raybaud F, Marchetto S, Planche J, Galland F, Mattei M-G, Copeland NG, Jenkins NA, Coulier F, Birnbaum D: Structure, chromosome mapping and expression of the murine FGF-6 gene. Oncogene 5:823–831, 1990.
94. Finch PW, Rubin JS, Miki T, Ron D, Aaronson SA: Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. Science 245:752–755, 1989.
95. Vlodavsky I, Fridman R, Sullivan R, Sasse J, Klagsbrun M: Aortic endothelial cells synthesize basic fibroblast growth factor which remains cell associated and platelet-derived growth factor-like protein which is secreted. J Cell Physiol 131:402–408, 1987.
96. Rogelj S, Weinberg RA, Fanning P, Klagsbrun M: Basic fibroblast growth factor fused to a signal peptide transforms cells. Nature 331:173–175, 1988.
97. Blam SB, Tischer E, Abraham JA, Aaronson S: Expression of acidic fibroblast growth factor in NIH/3T3 cells with and without the addition of a secretion signal sequence. J Cell Biochem (Suppl. 13b):152, 1989.
98. Rogelj S, Klagsbrun M: Oncogenic transformation by basic fibroblast growth factor. In: Krey LC, Gulyas BJ, McCracken JA (eds): Autocrine and Paracrine Mechanisms in Reproductive Endocrinology. Plenum, New York, 1989, pp 19–27.
99. D'Amore PA, Klagsbrun M: Angiogenesis: Factors and mechanisms. In: Sirica AE (ed): The Pathobiology of Neoplasia. Plenum, New York, 1989, pp 513–531.
100. Folkman J: What is the evidence that tumors are angiogenesis dependent? J Natl Cancer Inst 82:4–6, 1990.
101. Folkman J: Successful treatment of an angiogenic disease. N Engl J Med 320:1211–1212, 1989.
102. Folkman J, Waston K, Ingber D, Hanahan D: Induction of angiogenesis during the transition from hyperplasia to enoplasia. Nature 339:58–61, 1989.
103. Paku S, Paweletz N: First steps of tumor-related angiogenesis. Lab Invest 65:334–346, 1991.
104. Brem H, Guerin C, Tamargo R, Brem S, Brem H: Brain tumor angiogenesis. In: Kornblith PL (ed): Advances in Neuro-oncology. Futura, Mount Kisco, NY, 1988, pp 89–101.
105. Blood CH, Zetter BR: Tumor interactions with the vasculature: Angiogenesis and tumor metastasis. Biochem Biophys Res Acta 1032:89–118, 1990.
106. Zetter BR: Angiogenesis: State of the art. Chest 93:159S–166S, 1988.
107. Moses MA, Langer R: A metalloproteinase inhibitor of neovascularization. J Cell Biochem 47:1–6, 1991.
108. Zagzag D, Brem S, Robert F. Neovascularization and tumor growth in the brain: A model for experimental studies of angiogenesis and the blood-brain barrier. Am J Pathol 131:361–372, 1988.
109. Bouck N: Tumor angiogenesis: The role of oncogenes and tumor suppressor genes. Cancer Cells 2:179–184, 1990.
110. Weidner N, Semple JP, Welch WR, Folkman J: Tumor angiogenesis and metastasis — correlation in invasive breast carcinoma. N Engl J Med 324:1–8, 1991.
111. Levy AP, Tamargo R, Brem H, Nathans D: An endothelial cell growth factor from the mouse neuroblastoma cell line NB41. Growth Factors 2:9–19, 1989.
112. Conn SC, Kamen R: Amino acid and cDNA sequences of a vascular endothelial cell mitogen that is homologous to platelet-derived growth factor. Proc Natl Acad Sci USA 87:2628–2632, 1990.
113. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, et al.: Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest 84:1478–1489, 1989.
114. Rosenthal RA, Megyesi JF, Henzel WJ, Ferrara N, Folkman J: Conditioned medium from mouse sarcoma 180 cells contains vascular endothelial growth factor. Growth Factors 4:53–59, 1990.

115. Shing Y, Folkman J, Haudenschild C, Lund D, Crum R, Klagsbrun M: Angiogenesis is stimulated by a tumor-derived endothelial cell growth factor. J Cell Biochem 29:275–287, 1985.
116. Lobb RR, Alderman EM, Fett JW: Induction of angiogenesis by bovine brain derived class I heparin-binding growth factor. Biochemistry 24:4969–4973, 1985.
117. Klagsbrun M, Vlodavsky I: Biosynthesis and storage of basic fibroblast growth factor (BFGF) by endothelial cells: Implication for the mechanism of action of angiogenesis. In: Barbul A, Pines E, Caldwell M, Hunt TK (eds): Progress in Clinical and Biological Research; Growth Factors and Other Aspects of Wound Healing.
118. Ishai-Michaeli R, Eldor A, Vlodavsky I: Heparanase activity expressed by platelets, neutrophils, and lymphoma cells releases active fibroblast growth factor from extracellular matrix. Cel Regul 1:833–842, 1990.
119. Voldavsky I, Fuks Z, Ishai-Michaeli R, Bashkin P, Levi E, Korner G, Bar-Shavit, Klagsbrun M: Extracellular matrix-resident basic fibroblast growth factor: Implication for the control of angiogenesis. J Cell Biochem 45:167–176, 1991.
120. Presta M, Maier JAM, Rusnati M, Ragnotti G: Basic fibroblast growth factor is released from endothelial extracellular matrix in a biologically active form. J Cell Physiol 140:68–74, 1989.
121. Gross JL, Herblin WF, Dusak BA, Czerniak P, Diamond M, Dexter DL: Modulation of solid tumor growth in vivo by bFGF. Proc Am Assoc Cancer Res, 31:79, 1990.
122. Tanaka A, Matsumoto K, Nishizawa Y, Lu J, Yamanishi H, Maeyama M, Nonomura N, Uchida N, Sato B: Growth stimulation by androgens, glucocorticoids or fibroblast growth factors and the blocking of the stimulated growth by antibody against basic fibroblast growth factor in protein-free culture of shionogi carcinoma 115 cells. J Steroid Biochem Mol Biol 37:23–29, 1990.
123. Hori A, Sasada R, Matsutani E, Naito K, Sakura Y, Fujita T, Kozai Y: Suppression of solid tumor growth by immunoneutralizing monoclonal antibody against human basic fibroblast growth factor. Cancer Res 51:6180–6184, 1991.
124. Kandel J, Bossy-Wetzel E, Radvanyi F, Klagsbrun M, Folkman J, Hanahan D: Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. Cell 66:1095–1104, 1991.
125. Becker D, Meier CB, Herlyn M: Proliferation of human malignant melanomas is inhibited by antisense oligodeoxynucleotides targeted against basic fibroblast growth factor. EMBO J 8:3685–3691, 1989.
126. Yayon A, Klagsbrun M: Autocrine transformation by chimeric signal peptide-basic fibroblast growth factor: Reversal by suramin. Proc Natl Acad Sci USA 87:5346–5350, 1990.
127. Moscatelli D, Quarto N: Transformation of NIH 3T3 cells with basic fibroblast growth factor or the hst/K-*fgf* oncogene causes downregulation of the fibroblast growth factor receptor: Reversal of morphological transformation and restoration of receptor number by suramin. J Cell Biol 109:2519–2527, 1989.
128. Rapraeger AC, Krufka A, Olwin BB: Requirement of heparan sulfate for bFGF mediated fibroblast growth and myoblast differentiation. Science 252:1705–1708, 1991.
129. Sakamoto N, Iwahana M, Tanaka NG, Osada Y: Inhibition of angiogenesis and tumor growth by a synthetic laminin peptide, CDPGYIGSR-NH_2. Cancer Res 51:903–906, 1991.
130. Ingber D, Fujita T, Koshitmoto S, Sudo K, Kanamaru T, Brem H, Folkman J: Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumor growth. Nature 348:555–557, 1990.
131. Kusaka M, Sudo K, Fujita T, Marui S, Itoh F, Ingber D, Folkman J: Potent anti-angiogenic action of AGM-1470: Comparison to the fumagillin parent. Biochem Biophys Res Commun 174:1070–1076, 1991.
132. Brem H, Ingber D, Blood C, Bradley BS, Urioste S, Folkman J: Suppression of tumor metastasis by angiogenesis inhibition. Surg Forum 17:439–441, 1991.
133. Brem H, Tsakayannis D, Folkman J: Time dependent suppression of wound healing with

the angiogenesis inhibitor, AGM 1470. J Cell Biol 115:403, 1991.
134. Tamargo RJ, Bok RA, Brem H: Angiogenesis inhibition by minocycline. Cancer Res 51:672–675, 1991.
135. Bagavandoss P, Wilks JW: Specific inhibition of endothelial cell proliferation by thrombospondin. Biochem Biophys Res Comm 170:867–972, 1990.
136. Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, Bouck NP: A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci USA 87:6624–6628, 1990.
137. Moses M, Sudhalter J, Langer R: Identification of an inhibitor of neovascularization from cartilage. Science 248:1408–1410, 1990.
138. Matsubara T, Saura R, Hirohata K, Ziff M: Inhibition of human endothelial cell proliferation in vitro and neovascularization in vivo by D-penicillamine. J Clin Invest 83:158–167, 1989.
139. Brem SS, Zagzag D, Tsanaclis AMC, Gately S, Elkouby M-P, Brien SE: Inhibition of angiogenesis and tumor growth in the brain: Suppression of endothelial cell turnover by penicillamine and the depletion of copper, an angiogenic cofactor. Am J Pathol 137:1121–1142, 1990.
140. Maione TE, Gray GS, Hunt AJ, Sharpe RJ: Inhibition of tumor growth in mice by an analogue of platelet factor 4 that lacks affinity for heparin and retains potent angiostatic activity. Cancer Res 51:2077–2083, 1991.
141. Ingber D, Folkman J: Inhibition of angiogenesis through modulation of collagen metabolism. Lab Invest 59:44–51.
142. Crum R, Szabo S, Folkman J: A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science 230:1375–1378, 1985.
143. Tamargo RJ, Leong KW, Brem H: Growth inhibition of the 9L glioma using polymers to release heparin and cortisone acetate. J Neurol Oncol 9:131–138, 1990.
144. Folkman J, Weisz PB, Joullie MM, Li WW, Ewing WR: Control of angiogenesis with synthetic heparin substitutes. Science 243:1490–1493, 1989.
145. Sano H, Forough R, Maier JAM, Case JP, Jackson A, Engleka K, Maciag T, Wilder RL: Detection of high levels of heparin binding growth factor-1 (acidic fibroblast growth factor) in inflammatory arthritic joints. J Cell Biol 110:1417–1426, 1990.
146. Sivalingam A, Kenney J, Brown GC, Benson WE, Donoso L: Basic fibroblast growth factor levels in the vitreous of patients with proliferative diabetic retinopathy. Arch Ophthalmol 108:869–872, 1990.
147. Chodak GW, Hospelhorn V, Judge SM, et al.: Increased levels of fibroblast growth factor-like activity in urine from patients with bladder or kidney cancer. Cancer Res 48:2083–2088, 1988.
148. Watanabe H, Hori A, Seno M, Kozai Y, Igarashi K, Ichimori Y, Kondo K: A sensitive enzyme immunoassay for human basic fibroblast growth factor. Biochem Biophys Res Commun 175:229–235, 1991.
149. Fujimoto K, Ichimori Y, Kakizoe T, Okajima E, Sakamoto H, Sugimura T, Terada M: Increased serum levels of basic fibroblast growth factor in patients with renal cell carcinoma. Biochem Biophys Res Commun 180:386–392, 1991.
150. Murphy PR, Myal Y, Sato Y, Sato R, West M, Friesen HG: Elevated expression of basic fibroblast growth factor messenger ribonucleic acid in acoustic neuromas. Mol Endocrinol 3:225–231, 1989.
151. Silverlight JJ, Prysor-Jones RA, Jenkins JS: Basic fibroblast growth factor in human pituitary tumors. Clin Endocrinol 32:669–676, 1990.
152. Donovan-Peluso M, Contento AM, Tobon H, Ripepi B, Locker J: Oncogene amplification in breast cancer. Am J Pathol 138:835–845, 1991.
153. Roux-Dosseto M, Martin PM: A paradigm for oncogene complementation in human breast cancer. Res Virol 140:571–591, 1989.
154. Tang R, Kacinski B, Validire P, Beuvon F, Sastre X, Benoit P, dela Rochefordiere A,

Mosseri V, Pouillart P, Scholl S: Oncogene amplification correlates with dense lymphocyte infiltration in human breast cancers: A role for hematopoietic growth factor release by tumor cells? J Cell Biochem 44:189–198, 1990.
155. Theillet C, Adnane J, Szepetowski P, Simon M-P, Jeanteur P, Birnbaum D, Gaudray P: *BCL*-1 participates in the 11q13 amplification found in breast cancer. Oncogene 5:147–149, 1990.
156. Matsuzaki K, Yoshitake Y, Miyagiwa M, et al.: Production of basic fibroblast growth factor-like factor by cultured human cholangiocellular carcinoma cells. Jpn Cancer Res 81:345–354, 1990.
157. Tiesman J, Rizzino A: Expression and developmental regulation of the k-FGF oncogene in human and murine embryonal carcinoma cells. In Vitro Cell Dev Biol 25:1193–1198, 1989.
158. Wagata T, Ishizaki K, Imamura M, Shimada Y, Ikenaga M, Tobe T: Deletion of 17p and amplification of the *int*-2 gene in esophageal carcinomas. Cancer Res 51:2113–2117, 1991.
159. Tsuda T, Tahara E, Kajiyama G, Sakamoto H, Terada M, Sugimura T: High incidence of coamplification of *hst*-1 and *int*-2 genes in human esophageal carcinomas. Cancer Res 49:5505–5508, 1989.
160. Moscatelli D, Presta M, Joseph-Silverstein J, Rifkin DB: Both normal and tumor cells produce basic fibroblast growth factor. J Cell Physiol 129:273–276, 1986.
161. Presta M, Moscatelli D, Silverstein JJ, Rifkin DB: Purification from a human hepatoma cell line of a basic fibroblast growth factor-like molecule that stimulates capillary endothelial cell plasminogen activator production, DNA synthesis and migration. Molec Cell Biol 6:4060–4066, 1986.
162. Klagsbrun M, Sasse J, Sullivan R, Smith JA: Human tumor cells synthesize an endothelial cell growth factor that is structurally related to basic fibroblast growth factor. Proc Natl Acad Sci USA 83:2448–2452, 1986.
163. Miller WH Jr., Moy D, Li A, Grippo JF, Dmitrovsky E: Retinoic acid induces down-regulation of several growth factors and proto-oncogenes in a human embryonal cancer cell line. Oncogene 5:511–517, 1990.
164. Schulze-Osthoff K, Goerdt S, Sorg C: Expression of basic fibroblast growth factor (bFGF) in Kaposi's sarcoma: An immunohistologic study. J Invest Dermatol 95:238–240, 1990.
165. Huang SS, Tsai CC, Adams SP, Huang JS: Neuron localization and neuroblastoma cell expression of brain-derived growth factor. Biochem Biophys Res Commun 144:81–87, 1987.
166. Sasano H, Garrett CT, Wilkinson DS, Silverberg S, Comerford J, Hyde J: Protooncogene amplification and tumor ploidy in human ovarian neoplasms. Human Pathol 21:382–391, 1990.
167. Beauchamp RD, Lyons RM, Yang EY, Coffey RJ, Jr., Moses HL: Expression of and response to growth regulatory peptides by two human pancreatic carcinoma cell lines. Pancreas 5:369–380, 1990.
168. Nakamoto T, Usui A, Oshima K, Ikemoto H, Mitani S, Usui T: Analysis of growth factors in renal cell carcinoma. Biochem Biophys Res Commun 153:818–824, 1988.
169. Adnane J, Gaudray P, Dionne CA, Crumley G, Jaye M, Schlessinger J, Jeanteur P, Birnbaum D, Theillet C: *BEK* and *FLG*, two receptors to members of the FGF family, are amplified in subsets of human breast cancers. Oncogene 6:659–663, 1991.
170. Paulus W, Grothe C, Sensenbrenner M, Janet T, Baur I, Graf M, Roggendorf W: Localization of basic fibroblast growth factor, a mitogen and angiogenic factor, in human brain tumors. Acta Neuropathol 79:418–423, 1990.
171. Takahashi JA, Mori H, Fukumoto M, Igarashi K, Jaye M, Oda Y, Kikuchi H, Hatanaka M: Gene expression of fibroblast growth factors in human gliomas and meningiomas: Demonstration of cellular source of basic fibroblast growth factor mRNA and peptide in tumor tissues. Proc Natl Acad Sci USA 87:5710–5714, 1990.
172. Megyesi JF, Klagsbrun M, Folkman J, Rosenthal RA, Brigstock DR: 18.4 kDa basic

fibroblast growth factor (bFGF) alone is in the conditioned medium from human glioblastoma cells that synthesize four bFGF isoforms: Evidence for autocrine stimulation by bFGF. J Cell Biol 115:419a, 1991.
173. Morrison RS, Gross JL, Herblin WF, Reilly TM, LaSala PA, Alterman RL, Moskal JR, Kornblith PL, Dexter DL: Basic fibroblast growth factor-like activity and receptors are expressed in a human glioma cell line. Cancer Res 50:2524–2529, 1990.
174. Sato Y, Murphy PR, Sato R, Friesen HG: Fibroblast growth factor release by bovine endothelial cells and human astrocytoma cells in culture is density dependent. Mol Endocrinol 3:744–748, 1989.
175. Corin SJ, Chen LC, Hamburger AW: Enhancement of anchorage-independent growth of a human adrenal carcinoma cell line by endogenously produced basic fibroblast growth factor. Int J Cancer 46:516–521, 1990.
176. Brookes S, Smith R, Casey G, Dickson C, Peters G: Sequence organization of the human *int*-2 gene and its expression in teratocarcinoma cells. Oncogene 4:429–436, 1989.
177. Osthoff-Schulze K, Risau W, Vollmer E, Sorg C: *In situ* detection of basic fibroblast growth factor by highly specific antibodies. Am J Pathol 137:85–92, 1990.
178. Lobb RR, Rybak SM, St. Clair DK, Fett JW: Lysates of two established human tumor lines contain heparin-binding growth factors related to bovine acidic brain fibroblast growth factor. Biochem Biophys Res Commun 139:861–867, 1986.
179. Matsuura N, Seo M, Murata A, Ohmachi Y, Morikawa Y, Kakudo K: Immunohistochemical analysis of basic fibroblast growth factor in the human gastrointestinal cancer (abstracts). J Cell Biol 115:420, 1991.
180. Schweigerer L, Neufeld G, Mergia A, Abraham JA, Fiddes JC, Gospodarowicz D: Basic fibroblast growth factor in human rhabdomyosarcoma cells: Implications for the proliferation and neovascularization of myoblast-derived tumors. Proc Natl Acad Sci USA 84:842–846, 1987.
181. Xerri L, Hassoun J, Planche J, Guigou V, Grob J-J, Parc P, Birnbaum D, deLapeyriere O: Fibroblast growth factor gene expression in AIDS-Kaposi's sarcoma detected by *in situ* hybridization. Am J Pathol 138:9–15, 1991.

11. Transforming growth factor-alpha and its role in neoplastic progression

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Introduction

The discovery of transforming growth factor alpha (TGF-α) stemmed from a finding by Todaro et al. [1] in the mid-1970s that several retrovirally transformed cell lines showed reduced surface binding of radiolabeled EGF. This led to an examination of media conditioned by these cells for factors that would bind the EGF receptor, thereby making it unavailable to the exogenously added ligand. Such a factor was identified in media conditioned by feline sarcoma virus-transformed fibroblasts and was therefore named *sarcoma growth factor* (SGF) [2]. Subsequent studies showed that SGF-like activity could be identified in media conditioned by a variety of chemically and retrovirally transformed cells, but not by their normal cell counterparts [3–5]. This, in turn, stimulated the belief that the production of SGF contributed to the development and/or maintenance of the transformed phenotype.

Support for the aforementioned hypothesis came from the demonstration that preparations of SGF were able to promote a reversible, phenotypic transformation of normal rat kidney (NRK) fibroblasts, as evidenced by the growth of treated cells in semisolid media (soft agar) and a loss of their normal contact inhibition [2,6]. This prompted a change in nomenclature from SGF to *transforming growth factor* (TGF). The purification and further characterization of TGF revealed that its activity (as measured by the transformation of NRK cells), in fact, reflected the synergistic actions of two distinct and unrelated proteins that were subsequently named TGF-α and TGF-β [7,8]. Although TGF-β would not by itself induce NRK colony formation in soft agar, it would significantly enhance colony formation induced by TGF-α, the EGF receptor-binding component. We now appreciate that the requirement for both proteins reflects the use of this particular cell transformation model. In other assays, or with certain other cell lines, either polypeptide alone can function as an agonist [9]. It should also be stressed that, as is so often the case, the choice of names for these molecules reflects the accidental choice of the assay system used in their initial discovery and characterization. Other growth factors, acting either

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alone or in combination, will induce phenotypic transformation of cultured cells. Hence, this assay is by no means specific for either TGF-α or TGF-β. Nevertheless, a variety of evidence summarized for TGF-α below further implicates this molecule in neoplastic progression.

Synthesis and processing of TGF-α

Structure of the mature, fully processed ligand

Early gel-filtration analyses of TGF-α-like activities produced by a variety of rodent or human cell types revealed significant size heterogeneity, with active species ranging from a 6-kDa form predominant in media conditioned by tumor cell lines [10] to a 30- to 40-kDa species present in the urine of patients with disseminated cancer [11]. With most sources, both small and large forms were coproduced in varying proportions. Upon purification and protein sequence analysis, the 6-kDa form was found to be a 50 amino acid polypeptide with approximately 30% sequence homology with mouse and human EGFs [12,13]. Of the conserved residues, six are cysteines whose spacing is also preserved when compared with EGFs. It was therefore predicted — and subsequently confirmed [14,15] — that TGF-α forms a compact, three-loop structure similar to that of EGF. It is apparently the preservation of this overall structure that allows TGF-α to bind to the EGF receptor, and it is through this receptor that its biological actions [for a recent review see ref. 16] are mediated. TGF-α binds to the EGF receptor with an affinity comparable to that of EGF [17], but in many assays TGF-α is the more potent ligand [18–21]. A recent report suggests that this may be explained by the fact that internalized TGF-α/receptor complexes are preferentially recycled to the cell surface, whereas EGF/receptor complexes are degraded [22].

EGF and TGF-α are members of a family of EGF receptor ligands that are recognized by the preserved spacing of six cysteine residues, with a consensus pattern $-CX_7CX_{4-5}CX_{10-13}CXCX_8C-$. Other members of the family include the heparin-binding ligands, amphiregulin [23] and HB-EGF [24], as well as polypeptides encoded by the genomes of certain pox family viruses. An example of the latter is vaccinia growth factor (VGF) encoded by vaccinia virus [25–27]. Additionally, as described below, a growing list of secreted or integral membrane proteins with diverse function have been found to contain EGF-like repeats. This extended family includes secreted proteases (e.g., components of the blood-clotting cascade [28]), the products of homeotic genes critical for development (e.g., *Notch of Drosophila melanogaster* [29] and *glp-1* [30] and *lin-12* [31] of *Caenorhabditis elegans*) and other cell-surface proteins (e.g., the low-density lipoprotein (LDL) receptor [32]). What the function of the EGF-like elements in these latter proteins is remains unknown, although at present there is no evidence to

suggest that they are released as bioactive peptides. A very recent report suggests that some of the EGF-like elements in the Notch protein are essential for interactions with Delta (another transmembrane protein) that are critical for proper development of neural and epidermal lineages in *Drosophila* [33].

The integral membrane glycoprotein, proTGF-α

The cloning of human [34] and rat [35] cDNAs revealed that the 50 amino acid form of TGF-α is synthesized as a portion of a larger polypeptide (proTGF-α). Microheterogeneity of transcript splicing creates two species of mRNA that encode two forms of proTGF-a, of 159 and 160 amino acids, respectively [36]. The presence within this precursor molecule of a second hydrophobic domain, in addition to the signal peptide at the NH_4-terminus, suggested that it might be an integral membrane protein with the mature, 50 amino acid sequence located in the extracellular portion. Other features of the precursor include the presence of a potential glycosylation site immediately following the signal peptide, and a short, cysteine-rich cytoplasmic domain of about 40 amino acids. A schematic of this molecule is shown in Fig. 1. Release of the mature, 50 amino acid sequence from proTGF-α would occur through the cleavage of alanine-valine bonds that are contained within the sequence Val-Ala-Ala-**Ala-Val**-Val at the NH_2-terminus, and within the similar sequence **Ala-Val**-Val-Ala-Ala at the COOH-terminus. This cleavage specificity, which is reminiscient of that of elastase enzymes [37], is unusual and is not typical of the processing of other growth factor/hormone precursors that frequently occurs at sites of dibasic residues [38–41]. The involvement of an unusual specificity may indicate that proteolytic processing of proTGF-α is a regulated activity (see below). Interestingly, the ecto-domain of the membrane proteoglycan betaglycan, a component of the TGF-β receptor system, can be released as a soluble protein, and a similar Ala-Val-Val sequence is found on the external side of the transmembrane [42]. Whether this corresponds to the actual cleavage site is currently unknown.

The essential features of this model have been confirmed experimentally. For example, biochemical analyses [43–45] and immunofluorescence [46] have localized proTGF-α to the plasma membrane in the predicted orientation [43]. Studies from several laboratories have shown that processing of proTGF-α is frequently inefficient and incomplete, thus resulting in the release of larger forms of TGF-α due to cleavage at the carboxyl terminus only [44,47,48]. These partially processed forms appear larger than predicted, on the basis of their sequence, due to heterogeneous *N*- and *O*-linked glycosylation, and likely correspond to the larger forms identified earlier by gel filtration. In at least some instances, these larger soluble forms can be processed to a relatively homogeneous product of approximately

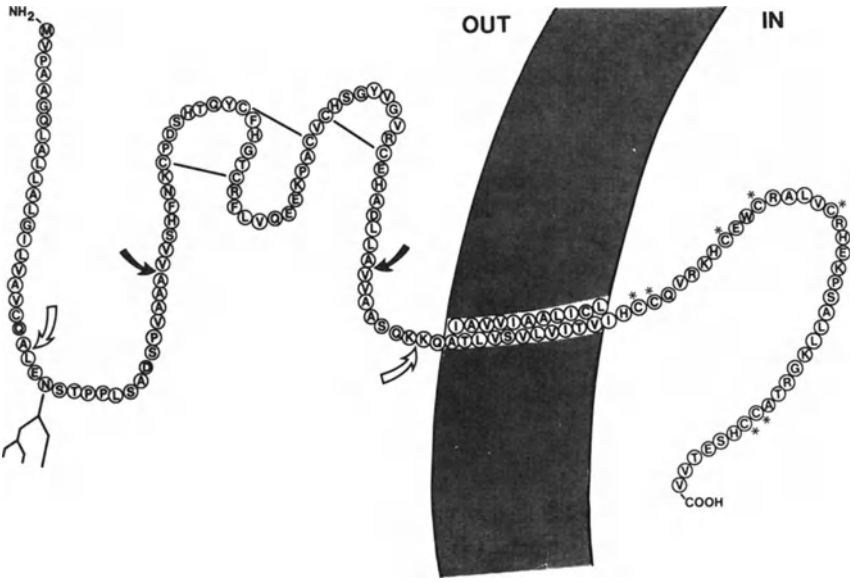

Figure 1. Model of the TGF-α precursor. The complete sequence of the 159 amino acid rat proTGF-α molecule is shown embedded in the plasma membrane. A consensus site for N-linked glycosylation is indicated, and cysteine residues within the cytoplasmic domain of the precursor are marked with asterisks. Darkened arrows indicate the amino acid bonds that must be proteolytically cleaved in order to release the mature, 50 amino acid growth factor. The open arrow near the NH_2-terminus designates the apparent signal peptide cleavage site between amino acid positions 22 and 23; however, our data suggest that cleavage of the signal peptide from proTGF-α is not invariant [43]. The open arrow immediately external to the plasma membrane marks a lysine–lysine bond that could serve as a potential cleavage site for a trypsinlike protease. In fact, we have failed to find evidence of cleavage at this site, even when high levels of exogenous trypsin are added to cells expressing proTGF-α [46].

6 kDa (the size of mature TGF-α) by treatment with pancreatic elastase [48,49].

Processing of proTGF-α

Relatively little is known about the in vivo processing of proTGF-α. Studies of Chinese hamster ovary (CHO) cells stably transfected with a proTGF-α expression vector have indicated that processing occurs in two steps after proTGF-α reaches the cell surface [50]. In the first step, which can be mimicked by exogenously added pancreatic elastase, proTGF-α rapidly (within minutes) loses the NH_2-terminal segment that precedes the mature sequence. The second step, which involves cleavage at the COOH-terminal site, occurs more slowly (within hours) and is not mimicked by exogenous elastase. Whether this differential cleavage reflects a requirement for two distinct enzymes is unknown, but it could account for the reported ac-

cumulation of proTGF-α species on the cell surface [51]. On the other hand, the kinetics of processing in CHO cells seem inconsistent with the commonly observed release of larger forms of TGF-α (see above). There are also conflicting data as to the requirement for glycosylation in the processing events. Whereas release of soluble TGF-α is not dependent on *N*- and *O*-glycosylation in transfected CHO cells, inhibitors of *N*-glycosylation interfere with TGF-α production in retrovirally transformed rat fibroblasts [47]. Thus, there may be some cell-type differences in the processing events. Finally, more recent studies suggest that cleavage of proTGF-α can be enhanced by various exogenous stimuli acting through protein kinase C-dependent and independent pathways [52,53]. These include phorbol esters, Ca^{2+} ionophores, and factors present in serum. These observations may be consistent with the regulation of processing.

The cleavage of TGF-α from an integral membrane precursor is analogous to the processing of other members of the EGF family of ligands, including (in addition to TGF-α and EGF) amphiregulin, HB-EGF, and VGF. In each case, the soluble ligand is released from a transmembrane protein precursor. The largest of these, proEGF, contains a total of nine EGF-like peptides in its external domain [54,55]. Only one of these — the peptide closest to the membrane — is known to be released as a bioactive ligand. The precursors to the other EGF family members are smaller and contain only one EGF-like unit each [24,34,35,56]. Except for the EGF-like elements, there are no other apparent sequence homologies between the various precursors. Additionally, there is no similarity with respect to the nature of those amino acids flanking the cleavage sites. However, incomplete or inefficient cleavage of the precursor molecules may be a recurrent theme, since proEGF is reported to accumulate in the distal tubules of the rodent kidney with little or no processing [57].

Juxtacrine actions of membrane-bound growth factors

Producing non cleavable forms of proTGF-α

The findings that proTGF-α is cleaved by a protease with unusual specificity and that, depending on the biological context, processing is not inevitable, raised the question as to whether the intact, membrane-bound precursor is biologically active. Could it bind to and activate EGF receptor located in adjacent or contiguous cell membranes? Such activity could not be taken for granted, in part because the precursor is relatively small (159–160 amino acids) and there is uncertainty as to how closely adjacent cells can approach one another. Additionally, it seemed possible that the bioactive domain, closely tethered to the membrane and immediately preceded by a glycosylation site, would be sterically hindered from binding to the receptor. Accordingly, our laboratory used site-directed mutagenesis to alter amino

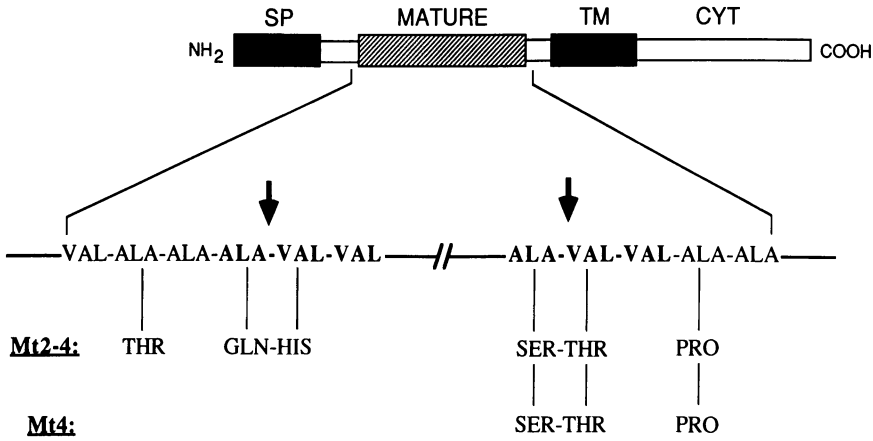

Figure 2. Schematic of the proTGF-α molecule and description of mutants. The various domains of the proTGF-α molecule are designated schematically. The sequence of amino acids that flank the mature growth factor in the rat proTGF-α molecule is shown below, with arrows marking the positions of the cleavage sites. The amino acid substitutions encoded by the two mutant constructs (Mt2–4 and Mt4) are indicated, along with the corresponding designations.

acid sequences flanking the proteolytic cleavage sites, and then characterized the biological activity of noncleavable forms of proTGF-α in transfected cells that do not otherwise produce the growth factor [46]. In carrying out these experiments, we also considered that proTGF-α could serve as a model for other integral membrane proteins that contain EGF-like sequences, including not only the precursors to the EGF family of ligands, but also the products of the aforementioned homeotic genes critical for early fly and worm development.

The nature of the mutations introduced into proTGF-α are shown in Fig. 2. Given the paucity of information regarding the specificity of the processing enzyme(s) and the fact that the flanking sequences contain additional valine and alanine residues, we were concerned about the possibility of residual cleavage in the flanking regions. We, therefore, prepared mutant forms of proTGF-α that not only contained substitutions of the dipeptides that comprise the amino- and carboxy-terminal cleavage sites, but also alterations in the flanking regions so that no val-ala or ala-ala dipeptides remained. This concern proved to be valid, since we subsequently determined that processing was blocked only in the case of the more extensively mutated molecules. We created two kinds of proTGF-α molecules — one altered so as to block processing at both termini (Mt2–4) and the other so as to prevent cleavage at the COOH-terminal site only (Mt4). This latter form would still anchor the mature growth factor sequence to the membrane, but would allow cleavage of the NH_2-terminal portion of the precursor. TGF-α cDNAs encoding either wild-type or mutant sequences were then cloned into an expression vector under the control of the zinc-

inducible mouse metallothionein promoter, and these constructs were transfected into cultured fibroblasts. Immunofluorescent staining of clones shown to be highly inducible for expression of both TGF-α mRNA and protein confirmed that both wild-type and mutant precursors were localized to the cell surface with the appropriate orientation. That the introduced mutations had indeed blocked processing of proTGF-α was shown by (1) gel analyses of immunoprecipitated, cell-associated TGF-α species in pulse-chase experiments and (2) examination of media conditioned by transfected cells using both Western blot analyses and sensitive bioassays that measure TGF-α's ability to compete with ^{125}I EGF for binding to the EGF receptor and to induce the growth of NRK cells in soft agar. The results of these various assays clearly revealed an absence of processing with both of the mutated forms of proTGF-α [46].

ProTGF-α is biologically active

The biological activity of noncleavable, mutant proTGF-α was examined by coincubation of zinc-induced cells with human A431 epidermoid carcinoma cells known to express high levels of EGF receptor [46]. The addition of cells bearing mutated proTGF-α on their surface rapidly induced an autophosphorylation of the A431 receptor that was not observed following the addition of either uninduced or parental (nontransfected) cells. To assay for receptor-mediated signal transduction, we looked for a rise in free, intracellular Ca^{2+}, a well-known early response to growth factor stimulation. Strikingly, the addition of cells bearing noncleavable proTGF-α produced a rapid (15–30 second) five-fold increase in the A431 intracellular Ca^{2+} levels. A significantly smaller rise followed the addition of uninduced cells and parental cells produced no response. Similar results were obtained using normal primary rat hepatocytes in place of A431 cells, and we further showed that the addition of plasma membranes bearing mutated proTGF-α (Mt2–4) to cultures of primary rat hepatocytes stimulated hepatocyte DNA synthesis up to four-fold (Fig. 3). In contrast, hepatocyte DNA synthesis was not stimulated by membranes from either uninduced or parental cells. Finally, we also examined the biological activity of intact proTGF-α by measuring its ability to transform NRK cells infected with retroviral expression vectors encoding either wild-type or noncleavable forms of proTGF-α [58]. We found that expression of both wild-type and mutant forms of proTGF-α transformed NRK cells as measured by their growth in soft agar and their ability to form tumors in nude mice. These results confirmed the biological activity of intact proTGF-α and indicate that growth factor sequence anchored to the membrane can productively interact with receptor in adjacent membranes. This type of ligand action has been termed *juxtacrine*, as opposed to the more familiar autocrine, paracrine, and endocrine actions. Our results also suggest that the accumulation of precursor on the surface of transformed cells is likely to have pathological

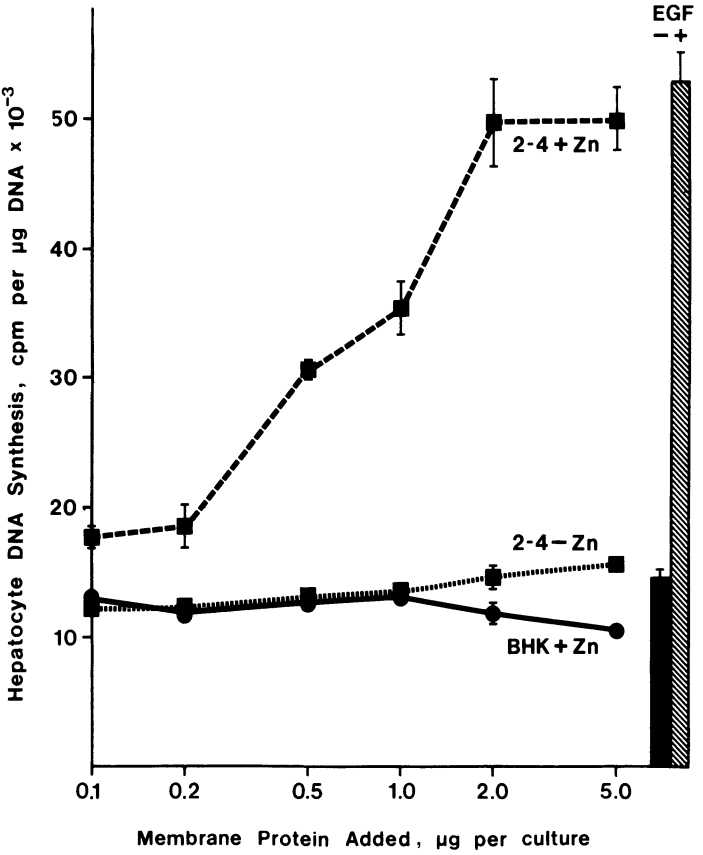

Figure 3. Dose curve of hepatocyte DNA synthesis in response to plasma membranes from BHK cells expressing a noncleavable form of proTGF-α. Plasma membranes were isolated from parental BHK cells (circles) and clones harboring constructs encoding the mutant, Mt2–4 form of proTGF-α (squares). Where indicated, cells were induced with zinc for 8 hours prior to harvesting, and plasma membranes were prepared according to established procedures. Increasing amounts of total membrane protein, together with 1 µCi of [^{3}H]-thymidine, were added to quiescent primary cultures of adult rat hepatocytes 6 hours after plating. Hepatocytes were harvested 48 hours later for scintillation counting and DNA assay. The level of DNA synthesis attained in the absence (solid bar) and presence (hatched bar) of EGF (10 ng/ml) is shown for comparison. Note that the induced Mt2–4 membranes containing the noncleavable proTGF-α stimulate hepatocyte DNA synthesis in a dose-dependent and saturable manner, and to a level comparable to that of EGF.

significance and that attempts to slow the growth of tumor cells by blocking the processing of proTGF-α may be ill founded.

Our conclusions regarding the biological activity of proTGF-α have been confirmed and extended by others. Derynck and colleagues used a similar mutagenesis strategy to express noncleavable forms of the human TGF-α precursor on the surface of transfected Chinese hamster ovary (CHO) cells. They then examined the activity of proTGF-α in cocultures with mouse

NIH3T3 fibroblasts that had been engineered to express high levels of EGF receptor. They showed that coincubation of the two cell types led to autophosphorylation of the NIH3T3 cell EGF receptor and that this activation induced c-*fos* expression [51]. Evidence suggests that preproEGF is also biologically active. Purified, wild-type human preproEGF bound and activated EGF receptor, and sustained the growth of EGF-dependent keratinocytes in culture [59]. Additionally, transfection of a preproEGF expression vector transformed NIH3T3 cells in culture [60]. Thus, biological activity of integral membrane precursors to growth factors may prove to be a generality. If so, this further implies that in contrast to the processing of soluble prohormones, which are generally inactive, cleavage of growth factor precursors may be aimed less at generating active molecules than at switching from membrane-anchored to diffusible forms.

Roles for membrane-bound growth factors in development?

The ability to restrict cleavage of precursor molecules and to limit growth-factor-mediated signalling to adjacent cells may be critical in certain biological contexts, for example, during development. A fascinating case in point is provided by the development of the compound eye in *Drosophila*, which is comprised of about 800 ommatidia or unit eyes [see ref. 61 and references contained therein]. Each of these, in turn, contains 20 distinct cells with eight photoreceptor neurons, together with several different non-neuronal cell types. An early step in the development of these structures from the eye imaginal discs is the assembly of cells into clusters. Within each cluster, the eight photoreceptor neurons differentiate stepwise in a stereotyped fashion that is apparently regulated by signals from adjacent cells. The best characterized event is the differentiation of the R7 photoreceptor cell, which occurs, at least in part, in response to a signal from the R8 cell. The induction of R7 can be blocked by mutations in any of several genes. One of these, *sevenless*, appears to encode a putative receptor with tyrosine kinase activity that is expressed on the surface of the R7 cell. Another critical gene, *bride of sevenless* (*boss*), encodes a protein with multiple, membrane-spanning domains that is believed to be the ligand for *sevenless*. In this context, it seems possible to imagine that the *sevenless* signal cannot be diffusable but instead must be tethered to the membrane and limited to the R7 cell. Interestingly, a more recent study [61] of *boss/sevenless* interaction addresses an important question, that is, how is the stimulation of receptor by membrane-anchored growth factor terminated? What is the equivalent of internalization and downregulation of soluble growth factor/receptor complex? In mixed cocultures of cells expressing, on the one hand, *sevenless*, and on the other, *boss*, *boss* immunoreactivity was internalized into the *sevenless* cells. Whether this is the result of *boss* being pulled out of its membrane, or whether binding somehow dictates cleavage of the extracellular sequences, is currently unknown.

Although the characterization of membrane-anchored forms of mammalian growth factors has not yet progressed to this elegant stage, evidence suggests that at least one is critical for normal development. The protooncogene, c-*kit*, is a receptor of the tyrosine kinase class whose normal signalling is essential for hematopoiesis, gametogenesis, and melanogenesis [62]. The c-*kit* ligand (*steel*) has recently been cloned and has been shown to be expressed in two alternate transmembrane forms as a result of tissue-specific, alternate splicing of transcripts. One of the transmembrane forms preferentially gives rise to soluble species, whereas the other remains cell surface associated. A particular mutant steel allele, Sl^d, impairs the development of hematopoietic cells, melanocytes, and germ cells. Significantly, analysis of this mutant revealed that it contains a deletion in the *steel* gene that removes the transmembrane and cytoplasmic domains [63]. One interpretation of this finding would be that the cell surface form of *steel* has a critical developmental role that cannot be supplanted by the soluble form that continues to be produced in Sl^d mice. Additional insights may come from recent advances in gene targeting, which might allow the development of lines of mice in which the membrane-anchored, but not soluble, forms of growth factors are eliminated.

Finally, that integral membrane precursors to growth factors might also play additional roles is supported by recent findings that they can simultaneously function as mediators of cell–cell adhesion and proliferation. For example, proTGF-α expressed in bone marrow-derived stromal cell monolayers binds to the EGF receptor on suspended hematopoietic progenitor cells, thereby promoting adhesion of the two cell types. Adhered progenitor cells form foci of sustained DNA replication and cellular proliferation [64]. Similarly, membrane-bound, pro-colony stimulating factor 1 (proCSF-1) expressed in fibroblast monolayers mediates the adhesion and proliferation of macrophages expressing the CSF-1 receptor [65]. Whether the integral membrane precursors have yet other roles — perhaps functioning as receptors as originally suggested by Pfeffer and Ullrich [66] — remains to be determined. In this regard, it is a curious observation that the COOH-terminal region of proTGF-α, which includes the transmenbrane and cytoplasmic domains (but not the receptor-binding sequence) is the most conserved (>95%) portion of the molecule [67].

Transcriptional regulation of TGF-α

Expression of TGF-α in normal and neoplastic cells

The availability of cDNA probes and specific antibodies has revealed that TGF-α expression is not restricted to neoplastic cells. During embryogenesis, TGF-α mRNA is present in the maternal rat decidua [68] and in the developing kidney, otic vesicle, oral cavity, pharyngeal pouch, and first and

second branchial arch of the mouse fetus at days 9 and 10 of gestation [69]. It has also been identified in preimplantation blastocysts by PCR methodology [70]. Finally, TGF-α protein and/or mRNA have been detected in various adult tissues, including pituitary [71], skin keratinocytes [72; with particularly high levels in psoriatic lesions; ref. 73], macrophages [74], regenerating liver [75], reproductive tissues [76,77], and mammary gland [78]. In adult rats, some of the highest levels of TGF-α mRNA are found in regions of the CNS [79–81], most notably the cervical spinal cord [81]. Although its precise physiological roles are not yet known, detection in these various tissues is perhaps consistent with postulated roles for the growth factor in such diverse processes as cell migration [19], wound healing [82], angiogenesis [20], and bone resorption [21].

The hallmark of TGF-α expression, however, is the finding that it is most prevalent and abundant in neoplastic cells and tissues. Thus, compared to normal tissue counterparts, the levels of TGF-α are consistently elevated in solid tumors as well as in cultured cells derived from these tumors [83–85]. In some cases, increased expression of TGF-α mRNA correlates with enhanced expression of EGF receptor [83]. Reflecting these observations, measurement of TGF-α protein in either urine or tumor effusions reportedly provides a useful marker in the clinical management of patients with hepatocellular carcinoma [86], and possibly for assessments of tumor burden and patient prognosis in the case of other cancers [87]. In culture, TGF-α expression is consistently elevated in cells transformed by chemicals [48,88] or by direct introduction of activated oncogenes [89,90]. A particularly convincing example is provided by the recent demonstration that expression of TGF-α mRNA is activated in cells that have been induced to express H-*ras* from an integrated vector under the control of the mouse metallothionein promoter [91]. That TGF-α functions as an autocrine factor to enhance the growth of some of the transformed cell lines that produce it has been experimentally verified in certain cases through the use of neutralizing antibodies [92].

In addition to the above-described transformation-associated regulation, the expression of TGF-α mRNA can also be induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in a chemically induced rat liver epithelial cell line [93], in cells of the bovine anterior pituitary gland [94], and in human keratinocytes [95]. This transient induction, which is partly mediated through increased transcription of the TGF-α gene [93], is distinguished from the classical TPA-mediated induction of either c-*myc* [96] or c-*fos* [97,98] by its delayed and prolonged time course, and by a possible requirement for early protein synthesis [93]. TGF-α mRNA can also be induced by EGF or TGF-α [72,93], itself, as well as by other hormones that may, like TPA, act through protein kinase C [93]. Finally, the expression of TGF-α mRNA in human breast cancer cells can also be induced by estrogens [99]. Although the molecular mechanisms responsible for regulation of the TGF-α gene have not yet been elucidated, a recent preliminary report

describes TPA and hormone-induced increases in bacterial chloramphenicol acetyl transferase (CAT) expression under the control of the human TGF-α promoter [100].

The TGF-α gene and its promoter

ProTGF-α is encoded by a 4.5–5.0 kb mRNA that contains relatively short and long 5′ and 3′ untranslated regions, respectively [36,101]. This transcript is, in turn, derived from a large gene that spans close to 100 kb of genomic DNA and is comprised of six exons, the first three of which are separated by large (30 kb) introns [36]. Interestingly, the 50 amino acid, EGF-like sequence is encoded by two exons (3 and 4), an organizational feature common to those EGF-like sequences that are known to be ligands (e.g., EGF, amphiregulin, VGF), but not those for which a ligand function has not been established (e.g., the remaining EGF-like repeats in preproEGF; see ref. 102). The human gene is located on the short arm of chromosome 2 in the p11-p13 region close to the breakpoint of the Burkitt's lymphoma t(2;8) variant [103]. Whether the latter observation has any significance with respect to the activation of TGF-α expression in neoplastic cells is unknown.

The TGF-α promoter has been identified in both human [104] and rat [36] genomic DNAs (Fig. 4). It is characterized by a G+C-rich sequence that is devoid of TATA or CAAT elements but contains multiple, potential recognition sites for other transcription factors, including Sp1 and E2F. Consistent with the absence of a TATA box, transcription of the rat promoter initiates from two major and multiple minor sites spanning over 200 bp of DNA. In contrast, transcription reportedly initiates from a single site in the human promoter. The basis for this discrepancy is unclear, since a sequence comparison reveals that the promoter is highly conserved between these species (Fig. 4). Working with the rat promoter, our laboratory has recently established a role for Sp1 in determining both the level of expression and in establishing the position of individual initiation sites [105]. On the other hand, we have thus far failed to demonstrate regulation of promoter-CAT constructs in transfected cells exposed to either TPA or estrogen, or in cells cotransfected with activated *ras* expression vectors. These findings suggest that regulation of TGF-α gene expression by these agents may not be mediated via direct response elements, but rather is indirect and possibly conferred through changes in the levels or activities of basal transcription factors.

Transforming properties of TGF-α in vivo

Creation of TGF-α transgenic mice

Studies from a number of laboratories have indicated that TGF-α (either exogenously provided or endogenously produced) can transform various

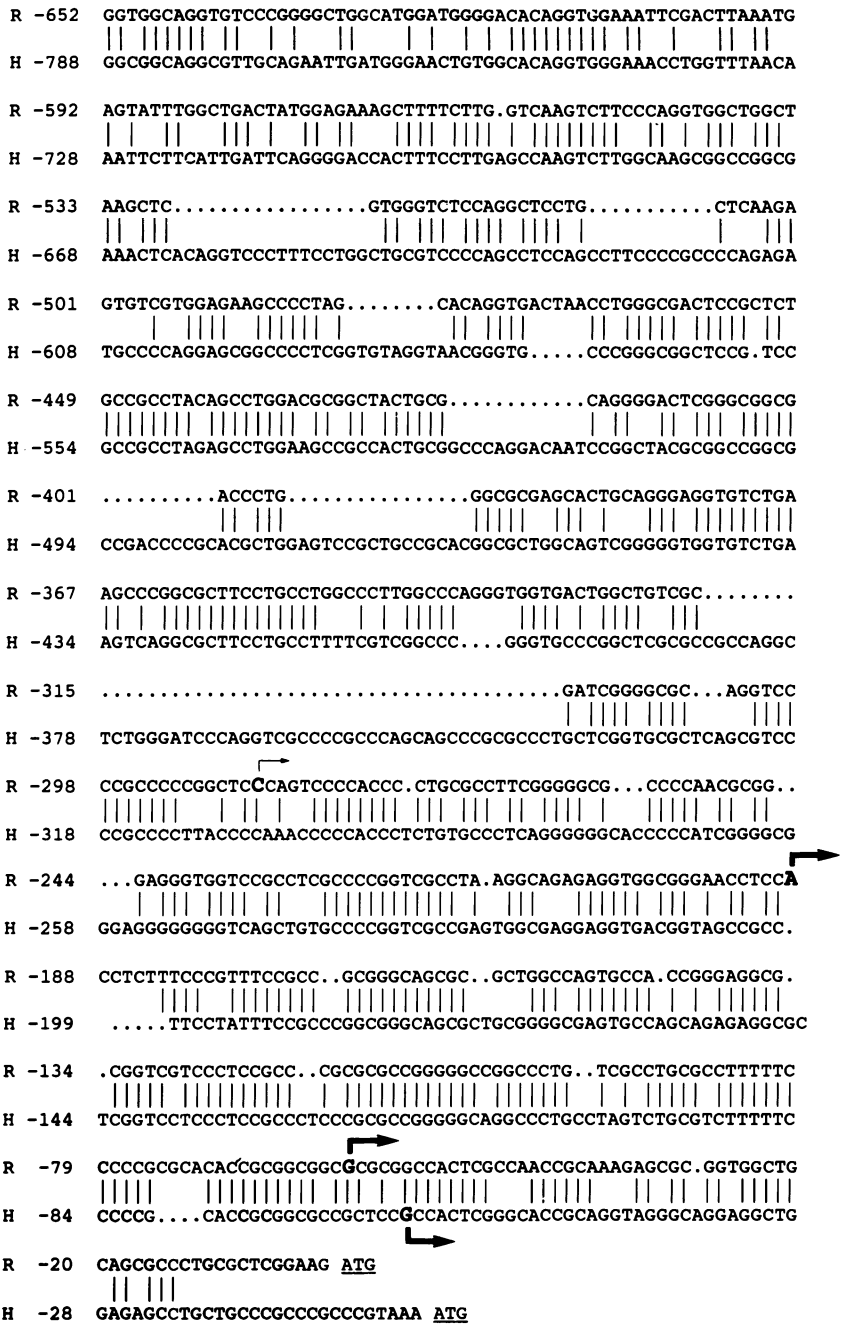

Figure 4. Nucleotide sequence comparison of the rat (above) and human (below) TGF-α promoters. In each case, nucleotide positions are numbered with respect to the first base of the codon, corresponding to the initiating AUG. Conserved nucleotides are marked, and gaps required for optimal alignment are indicated. The positions of the single human, and two predominant rat, transcription start sites are marked by heavy arrows; the position of the 5′-most minor rat transcription start site is indicated by a light arrow. The nucleotide sequences of the human and rat promoters are derived from refs. 104 and 36, respectively.

cells in culture [58,92,106]. While significant, a more important consideration relates to the possible extent to which deregulated expression of TGF-α contributes to the development and/or progression of neoplasia in vivo, particularly in the context of epithelial tissues from which most cancers arise. To address this issue, several laboratories have recently created lines of transgenic mice in which overexpression of proTGF-α is targeted to either specific or multiple tissues under the control of heterologous promoters. For example, we prepared expression vectors containing the TGF-α sequence under the control of either the metal-inducible mouse metallothionein I promoter, which is expressed in multiple tissues at various stages of development, or other promoters that restrict expression to specific tissues. In light of growing evidence that intron/exon structure is essential for efficient expression of transgenes in vivo [107], these constructs were designed either as proTGF-α cDNA/human growth hormone gene fusions (with growth hormone not expressed), or as proTGF-α cDNA/gene ('minigene') chimeras (since the normal TGF-α gene is too large to incorporate into a vector). These various constructs were then used to generate multiple lines of transgenic mice according to established procedures in a collaboration with Eric Sandgren, Ralph Brinster, and Richard Palmiter [108]. Subsequent analyses documented the apropriate expression of transgenic TGF-α mRNA and protein in various tissues.

Results obtained with our various lines of mice were mutually consistent and corroborative, and revealed a spectrum of tissue-specific responses in mature animals. For example, kidney generally showed no phenotype despite high levels of TGF-α expression and the presence of EGF receptor. On the other hand, some tissues, including liver and other parts of the gastrointestinal tract (e.g., colon), were markedly increased in size (with wet weight increases of two- to three-fold), but normal tissue architecture was preserved. Consistent with a hyperplastic basis, increased tissue mass was generally accompanied by similar relative increases in DNA content, indicating that TGF-α is a potent mitogen for these tissues and can play a role in establishing the 'set point' with respect to tissue size.

In certain other tissues, however, overexpression of TGF-α produced abnormal growth. For example, the pancreas was greatly enlarged (up to ten-fold) due to acinar cell and especially fibroblast proliferation, and showed marked evidence of acinar cell metaplasia with the frequent appearance of ductlike elements. The latter are reminiscient of ductlike structures (pseudoducts) that reportedly arise in the rodent pancreas in response to treatment with carcinogens [109] and that are also described in studies of human pancreatic cancer. Although, in the latter context these are thought to represent preneoplastic lesions, we have not observed the formation of pancreatic tumors, even in aged mice. The pancreatic phenotype was also reproduced in mice harboring a construct in which the proTGF-α sequence was placed under the control of the elastase promoter that specifically restricts expression to the acinar cells of the exocrine pancreas. That we saw

no effects in other tissues of these latter mice suggests that the growth factor's actions are largely local, i.e., autocrine and paracrine. Interestingly, the acinar cell metaplasia, but not the fibroplasia, was observed in the pancreases of mice that expressed the mutated, noncleavable form of proTGF-α, indicating that induction of fibroblast proliferation requires diffusion of the soluble growth factor. This result confirms the absence of processing of the mutated precursor in vivo. Finally, in addition to the aforementioned effects in the pancreas, one line of mice displayed growth abnormalities in the coagulation gland epithelium (the functions of which are partially subsumed by the prostate in men), with dramatic hyperplasia and dysplasia, and examples of carcinoma in situ.

Through the mating of different lines of transgenic mice, we are currently exploring the ways in which coexpression of TGF-α and certain oncoproteins alters the development of tumors in tissues, such as the pancreas, in which TGF-α does not itself act as a direct oncoprotein. Preliminary results suggest that coexpression of TGF-α dramatically accelerates the growth of tumors induced by several oncogenes, thus producing significant decreases in survival time.

TGF-α produces carcinoma of the breast

A consistent observation among our multiple lines of mice has been the development of breast cancers in mature females that have undergone one or more rounds of pregnancy [108]. This response was first noted in those animals in which expression is under the control of the metallothionein promoter, despite the fact that expression in the mammary tissue of these animals is significantly lower than that in other tissues. It has been preliminarily reproduced in mice expressing TGF-α transgenes in mammary epithelium under the control of the whey acidic protein (WAP) promoter, which specifically directs expression to the milk-producing alveolar cells from late pregnancy through lactation, as well as the mouse mammary tumor virus (MMTV) promoter, which additionally directs expression to ductal cells. In the case of the metallothionein-TGF-α mice, the development of the disease is generally characterized by the appearance of many (100 or more) hyperplastic alveolar nodules (HANs) at 10–18 months of age. These range in appearance from relatively normal but nonregressed glands to enlarged, dysplastic nodules. This is often followed by the coincidental and focal appearance of from one to several tumors per mammary pad. At least some of these tumors, which include examples of fibrotic tumors, as well as tumors with papillary or glandular architecture, can be classified as carcinomas, since they produce tumors upon subcutaneous injection of dispersed cells into syngeneic mice. Additionally, we have observed metastases to lung in some of the animals harboring breast tumors.

Based on the above findings, our working model is that the HANs correspond to preneoplastic lesions and that our transgenic mice provide a

model of neoplastic progression that culminates in the appearance of frank breast tumors. We conclude that TGF-α can act as an initiator of neoplastic progression in the mammary gland, though the focal nature of the tumor development suggests that other, critical genetic lesions are also required. Possibly, in the context of hormonal stimulation, the abnormal proliferation triggered by TGF-α in some way promotes the occurrence of these genetic lesions. We are currently assessing the validity of this model, in part by screening for altered expression and/or mutation of gene products previously implicated in the genesis of breast cancer, including members of the FGF family of growth factors, the EGF receptor, the related *neu*, and p53.

Other laboratories have coincidentally reached similar conclusions using transgenic mouse models. Coffey et al. have likewise noted the development of breast tumors in response to MMTV-directed expression of TGF-α [109]. Merlino and coworkers have reported the development of pancreatic lesions in metallothionein-TGF-α mice, similar to those described above [110]. They also report a relatively high incidence of hepatocellular carcinomas in older transgenic animals. The fact that we have only rarely observed hepatocellular carcinomas, even in older animals, may be attributed to the use of different lines of mice, since hybrid (C57Bl/6 × SJL) used in our studies has a relatively low incidence of spontaneous liver neoplasia.

Conclusions

Although the preceding discussion is focused largely on our own studies, work from numerous laboratories over the past several years has generated a significant body of knowledge regarding the expression, structure, and processing of TGF-α. Studies of proTGF-α have helped to confirm the juxtacrine hypothesis, and this molecule may prove to be a useful model in studies of membrane-anchored forms of growth factors. An addition, we have acquired some knowledge of the structure of the TGF-α gene and its promoter but are ignorant of the mechanisms that regulate its expression. Finally, a large body of evidence suggests that TGF-α can transform cells in culture, but much less is known about the ways in which TGF-α contributes to the development and progression of neoplastic disease. Hopefully, the development of transgenic models will not only help to fill this latter gap, but will also provide generally useful models of tumor progression in vivo.

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References

1. Todaro GJ, DeLarco JE, Cohen S: Transformation by murine and feline sarcoma viruses specifically blocks binding of epidermal growth factor to cells. Nature 264:26–31, 1976.
2. DeLarco JE, Todaro GJ: Growth factors from murine sarcoma virus-transformed cells. Proc Natl Acad Sci USA 75:4001–4005, 1978.
3. Todaro GJ, Fryling C, DeLarco JE: Transforming growth factors produced by certain human tumor cells: Polypeptides that interact with epidermal growth factor receptors. Proc Natl Acad Sci USA 77:5258–5262, 1980.
4. Roberts AB, Lamb LC, Newton DL, Sporn MB, DeLarco JE, Todaro GJ: Transforming growth factors: Isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. Proc Natl Acad Sci USA 77:3493–3498, 1980.
5. Ozanne B, Fulton RJ, Kaplan PL: Kirsten murine sarcoma virus transformed cell lines and a spontaneously transformed rat cell line produce transforming factors. J Cell Physiol 105:163–180, 1980.
6. Roberts AB, Frolik CA, Anzano MA, Sporn MB: Transforming growth factors from neoplastic and non-neoplastic tissues. Fed Proc 42:2621–2625, 1983.
7. Anzano MA, Roberts AB, Meyers CA, Komoriya A, Lamb LC, Smith JM, Sporn MB: Synergistic interactions of two classes of transforming growth factors from murine sarcoma cells. Cancer Res 42:4776–4778, 1982.
8. Anzano MA, Roberts AB, Smith JM, Sporn MB, DeLarco JE: Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type α and type β transforming growth factors. Proc Natl Acad Sci USA 80:6264–6268, 1983.
9. Roberts AB, Anzano MA, Wakefield LM, Roche NS, Stern DF, Sporn MB: Type β transforming growth factor: A bifunctional regulator of cellular growth. Proc Natl Acad Sci USA 82:119–123, 1985.
10. Marquardt H, Hunkapiller MW, Hood LE, Twardzik DR, DeLarco JE, Stephenson JR, Todaro GJ: Transforming growth factors produced by retrovirus-transformed rodent cells and human melanoma cells: Amino acid sequence homology with epidermal growth factor. Proc Natl Acad Sci USA 80:4684–4688, 1983.
11. Sherwin SA, Twardzik DR, Bohn WH, Cockley KD, Todaro GJ: High molecular weight transforming growth factor activity in the urine of patients with disseminated cancer. Cancer Res 43:403–407, 1983.
12. Marquardt H, Todaro GJ: Human transforming growth factor: Production by a melanoma cell line, purification and initial characterization. J Biol Chem 257:5220–5225, 1982.
13. Marquardt H, Hunkapiller MW, Hood LE, Todaro GJ: Rat transforming growth factor type 1: Structure and relation to epidermal growth factor. Science 223:1079–1082, 1984.
14. Kohda D, Shimada I, Miyake T, Fuwa T, Inagaki F: Polypeptide chain fold of human transforming growth factor α analagous to those of mouse and human epidermal growth factors as studied by two-dimensional ^{1}H NMR. Biochemistry 28:953–958, 1989.
15. Harvey TS, Wilkinson AJ, Tappin MJ, Cooke RM, Campbell ID: The solution structure of human transforming growth factor alpha. Eur J Biochem 198:555–562, 1991.
16. Carpenter G, Wahl MI: The epidermal growth factor family. In: Sporn MB, Roberts AB, (eds). Peptide Growth Factors and their Receptors I. Springer-Verlag, Berlin, 1990, pp 69–171.
17. Massague J: Epidermal growth factor-like transforming growth factor. II. Interaction with epidermal growth factor receptors in human placenta membranes and A431 cells. J Biol Chem 258:13614–13620, 1983.
18. Myrdal SE, Twardzik DR, Auersperg N: Cell-mediated co-action of transforming growth factors; incubation of type β with normal rat kidney cells produces a soluble activity that prolongs the ruffling response to type α. J Cell Biol 102:1230–1234, 1986.
19. Barrandon Y, Green H: Cell migration is essential for sustained growth of keratinocyte colonies: The roles of transforming growth factor-α and epidermal growth factor. Cell 50:1131–1137, 1987.

20. Schreiber AB, Winkler ME, Derynck R: Transforming growth factor-α: A more potent angiogenic mediator than epidermal growth factor. Science 232:1250–1253, 1986.
21. Ibbotson KJ, Harrod J, Gowen M, D'Souza S, Smith DD, Winkler ME, Derynck R, Mundy GR: Human recombinant transforming growth factor α stimulates bone resorption and inhibits formation *in vitro*. Proc Natl Acad Sci USA 83:2228–2232, 1990.
22. Ebner R, Derynck R: Epidermal growth factor and transforming growth factor-α: Differential intracellular routing and processing of ligand-receptor complexes. Cell Regul 2:599–612, 1991.
23. Schoyab M, Plowman GD, McDonald VL, Bradley JG, Todaro GJ: Structure and function of human amphiregulin: A member of the epidermal growth factor family. Science 243:1074–1076, 1989.
24. Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsburn M: A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. Science 251:936–939, 1991.
25. Blomquist MC, Hunt LT, Barker WC: Vaccinia virus 19-kilodalton protein: Relationship to several mammalian proteins, including two growth factors. Proc Natl Acad Sci USA 81:7363–7367, 1984.
26. Stroobant P, Rice AP, Gullick WJ, Cheng DJ, Kerr IM, Waterfield MD: Purification and characterization of a vaccinia virus growth factor. Cell 42:383–393, 1985.
27. Brown JP, Twardzik DR, Marquardt H, Todaro GJ: Vaccinia virus encodes a peptide homologous to epidermal growth factor and transforming growth factor. Nature 313:491–492, 1985.
28. Kurachi K, Davie EW: Isolation and characterization of a cDNA coding for factor IX. Proc Natl Acad Sci USA 79:6461–6464, 1982.
29. Wharton KA, Johansen KM, Xu T, Artavanis-Tsakonas S: Nucleotide sequence from the neurogenic locus *Notch* implies a gene product that shares homology with proteins containing EGF-like repeats. Cell 43:567–581, 1991.
30. Yochem J, Greenwald I: *glp-1* and *lin-12*, genes implicated in distinct cell-cell interactions in *C. elegans*, encode similar transmembrane proteins. Cell 58:553–563, 1989.
31. Greenwald I: *lin-12*, a nematode homeotic gene, is homologous to a set of mammalian proteins that includes epidermal growth factor. Cell 43:583–590.
32. Russell DW, Schneider WJ, Yamamoto T, Luskey KL, Brown MS, Goldstein JL: Domain map of the LDL receptor: Sequence homology with the epidermal growth factor precursor. Cell 37:577–585, 1984.
33. Rebay I, Fleming RJ, Fehon RG, Cherbas L, Cherbas P, Artavanis-Tsakonas S: Specific EGF repeats of *Notch* mediate interactions with *Delta* and *Serrate*: Implications for *Notch* as a multifunctional receptor. Cell 67:687–699, 1991.
34. Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV: Human transforming growth factor-α: Precursor structure and expression in *E. coli*. Cell 38:287–297, 1984.
35. Lee DC, Rose TM, Webb NR, Todaro GJ: Cloning and sequence analysis of a cDNA for rat transforming growth factor-α. Nature 313:489–491, 1985.
36. Blasband AJ, Rogers KT, Chen X, Azizkhan JC, Lee DC: Characterization of the rat transforming growth factor-α gene and identification of promoter sequences. Mol Cell Biol 10:2111–2121, 1990.
37. Naughton MA, Sanger F: Purification and specificity of pancreatic elastase. Biochem J 78:156–163, 1961.
38. Noda M, Furatani Y, Takahashi H, Toyosato M, Hirose T, Imayama S, Nakanishi S, Numa S: Cloning and sequence analysis of bovine adrenal preproenkephalin. Nature 295:202–206, 1982.
39. Gubler U, Seeburg P, Hoffman BJ, Gage LP, Udenfriend S: Molecular cloning establishes pro-enkephalin as a precursor of enkaphalin-containing peptides. Nature 295:206–208, 1982.
40. Amara SG, Jonas V, Rosenfeld MG, Ong ES, Evans RM: Alternative RNA processing in

calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature 298:240–244, 1982.
41. Nakanishi S, Inoue E, Nakamura M, Change ACY, Cohen SN, Numa S: Nucleotide sequence of cloned cDNA for bovine corticotropin-b-lipotropin precursor. Nature 278:423–427, 1979.
42. Lopez-Casillas F, Cheifetz S, Doody J, Andres JL, Lane WS, Massague J: Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-β receptor system. Cell 67:785–795, 1991.
43. Teixido J, Gilmore R, Lee DC, Massague J: Integral membrane glycoprotein properties of the prohormone pro-transforming growth factor-α. Nature 326:883–885, 1987.
44. Bringman TS, Lindquist PB, Derynck R: Different transforming growth factor-α species are derived from a glycosylated and palmitoylated transmembrane precursor. Cell 48:429–440, 1987.
45. Gentry LE, Twardzik DR, Lim GJ, Ranchalis JE, Lee DC: Expression and characterization of transforming growth factor α precursor protein in transfected mammalian cells. Mol Cell Biol 7:1585–1591, 1987.
46. Wong ST, Winchell LF, McCune BK, Earp HS, Teixido J, Massague J, Herman B, Lee DC: The TGF-α precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. Cell 56:495–506, 1989.
47. Teixido J, Massague JM: Structural properties of a soluble bioactive precursor for transforming growth factor-α. J Biol Chem 263:3924–3929, 1988.
48. Luetteke NC, Michalopoulos GK, Teixido J, Gilmore R, Massague J, Lee DC: Characterization of high molecular weight transforming growth factor α produced by rat hepatocellular carcinoma cells. Biochemistry 27:6487–6494, 1988.
49. Ignotz RA, Kelly B, Davis RJ, Massague J: Biologically active precursor for transforming growth factor type α released by retrovirally transformed cells. Proc Natl Acad Sci USA 83:6307–6311, 1986.
50. Teixido J, Wong ST, Lee DC, Massague J: Generation of transforming growth factor-α from the cell surface by an *O*-glycosylation-independent multistep process. J Biol Chem 265:6410–6415, 1990.
51. Brachmann R, Lindquist PB, Nagashima M, Kohr W, Lipari T, Napier M, Derynck R: Transmembrane TGF-α precursors activate EGF/TGFa receptors. Cell 56:691–700.
52. Pandiella A, Massague J: Cleavage of the membrane precursor for transforming growth factor α is a regulated process. Proc Natl Acad Sci USA 88:1726–1730, 1991.
53. Pandiella A, Massague J: Multiple signals activate cleavage of the membrane transforming growth factor-α precursor. J Biol Chem 266:5769–5773, 1991.
54. Gray A, Dull TJ, Ullrich A: Nucleotide sequence of epidermal growth factor cDNA predicts a 128,000-molecular weight protein precursor. Nature 303:722–725, 1983.
55. Scott J, Urdea M, Quiroga M, Sanchez-Pescador R, Fong N, Selby M, Rutter WJ, Bell GI: Structure of a mouse submaxillary gland messenger RNA encoding epidermal growth factor and seven related proteins. Science 221:236–240, 1983.
56. Plowman GD, Green JM, McDonald VL, Neubauer MG, Disteche CM, Todaro GJ, Shoyab M: The amphiregulin gene encodes a novel epidermal growth factor-related protein with tumor-inhibitory activity. Mol Cell Biol 10:1969–1981, 1990.
57. Rall LB, Scott J, Bell GI, Crawford RJ, Penschow JD, Niall HD, Coghlan JP: Mouse prepro-epidermal growth factor synthesis by kidney and other tissues. Nature 313:228–231, 1985.
58. Blasband AJ, Gilligan DM, Winchell LF, Wong ST, Luetteke NC, Rogers KT, Lee DC: Expression of the TGFα integral membrane precursor induces transformation of NRK cells. Oncogene 5:1213–1221, 1990.
59. Mroczkowski B, Reich M, Chen K, Bell GI, Cohen S: Recombinant human epidermal growth factor precursor is a glycosylated membrane protein with biological activity. Mol Cell Biol 9:2771–2778, 1989.

60. Heidaran MA, Fleming TP, Bottaro DP, Bell GI, Di Fiore PP, Aaronson SA: Transformation of NIH3T3 fibroblasts by an expression vector for the human epidermal growth factor precursor. Oncogene 5:1265–1270, 1990.
61. Kramer H, Cagan RL, Zipursky L: Interaction of *bride of sevenless* membrane-bound ligand and the *sevenless* tyrosine-kinase receptor. Nature 352:207–212, 1991.
62. Yarden Y, Kuang W-J, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, Chen E, Schlessinger J, Francke U, Ullrich A: Human proto-oncogene c-*kit*: A new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J 6:3341–3351, 1987.
63. Flanagan JG, Chan DC, Leder P: Transmembrane form of the *kit* ligand growth factor is determined by alternative splicing and is missing in the Sl^d mutant. Cell 64:1025–1035, 1991.
64. Anklesaria P, Teixido J, Laiho M, Pierce JH, Greenberger JS, Massague J: Cell-cell adhesion mediated by binding of membrane-anchored transforming growth factor to epidermal growth factor receptors promotes cell proliferation. Proc Natl Acad Sci USA 87:3289–3293, 1990.
65. Stein J, Borzillo GV, Rettenmier CW: Direct stimulation of cells expressing receptors for macrophage colony-stimulating factor (CSF-1) by a plasma membrane-bound precursor of human CSF-1. Blood 76:1308–1314, 1990.
66. Pfeffer S, Ullrich A: Epidermal growth factor: Is the precursor a receptor? Nature 313:184, 1985.
67. Todaro GJ, Lee DC, Webb NR, Rose TM, Brown JP: Rat type α transforming growth factor: Structure and possible function as a membrane receptor. Cancer Cells 3:51–58, 1985.
68. Han VKM, Hunter ES III, Pratt RM, Zendegui JG, Lee DC: Expression of rat transforming growth factor alpha mRNA during development occurs predominantly in the maternal decidua. Mol Cell Biol 7:2335–2343, 1987.
69. Wilcox JN, Derynck R: Developmental expression of transforming growth factors alpha and beta in mouse fetus. Mol Cell Biol 8:3415–3422, 1988.
70. Rappollee DA, Brenner CA, Schultz R, Mark D, Werb Z: Developmental expression of PDGF, TGF-α, and TGF-β genes in preimplantation mouse embryos. Science 241:1823–1825, 1988.
71. Samsoondar J, Kobrin MS, Kudlow JE: α-transforming growth factor secreted by untransformed bovine anterior pituitary cells in culture I. J Biol Chem 261:14408–14413, 1986.
72. Coffey RJ, Jr., Derynck R, Wilcox JN, Bringman TS, Goustin AS, Moses HL, Pittelkow MR: Production and auto-induction of transforming growth factor-α in human keratinocytes. Nature 328:817–820, 1987.
73. Elder JT, Fisher GJ, Lindquist PB, Bennett GL, Pittelkow MR, Coffey RJ, Jr., Ellingsworth L, Derynck R, Voorhees JJ: Overexpression of transforming growth factor α in psoriatic epidermis. Science 243:811–814, 1989.
74. Madtes DK, Raines EW, Sakariassen KS, Assoian RK, Sporn MB, Bell GI, Ross R: Induction of transforming growth factor-α in activated human alveolar macrophages. Cell 53:285–293, 1988.
75. Mead J, Fausto N: Transforming growth factor α may be a physiological regulator of liver regeneration by means of an autocrine mechanism. Proc Natl Acad Sci USA 86:1558–1562, 1989.
76. Lobb DK, Kobrin MS, Kudlow JE, Dorrington JH: Transforming growth factor-alpha in the adult bovine ovary: Identification in growing ovarian follicles. Biol Reprod 40:1087–1093, 1989.
77. Skinner MK, Takacs K, Coffey RJ: Transforming growth factor-α gene expression and action in the seminiferous tubule: Peritubular cell-Sertoli cell interactions. Endocrinology 124:845–854, 1989.
78. Liscia DS, Merlo G, Ciardiello F, Kim N, Smith GH, Callahan R, Salomon DS:

Transforming growth factor-α messenger RNA localization in the developing adult rat and human mammary gland by *in situ* hybridization. Dev Biol 140:123–131, 1990.
79. Wilcox JN, Derynck R: Localization of cells synthesizing transforming growth factor-alpha mRNA in the mouse brain. J Neurosci 8:1901–1904, 1988.
80. Kudlow JE, Leung AWC, Kobrin MS, Paterson AJ, Asa SL: Transforming growth factor-α in the mammalian brain. Immunohistochemical detection in neurons and characterization of its mRNA. J Biol Chem 264:3880–3883, 1989.
81. Seroogy KB, Han VKM, Lee DC: Regional expression of transforming growth factor-α mRNA in the rat central nervous system. Neurosci Lett 125:241–245, 1991.
82. Schultz GS, White M, Mitchell R, Brown G, Lynch J, Twardzik DR, Todaro GJ: Epithelial wound healing enhanced by transforming growth factor-α and vaccinia growth factor. Science 235:350–352, 1987.
83. Derynck R, Goeddel DV, Ullrich A, Gutterman JU, Williams RD, Bringman TS, Berger WH: Synthesis of mRNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumors. Cancer Res 47:707–712, 1987.
84. Smith JJ, Derynck R, Korc M: Production of transforming growth factor α in human pancreatic cancer cells: Evidence for a superagonist autocrine cycle. Proc Natl Acad Sci USA 84:7567–7570, 1987.
85. Mydlo JH, Michaeli J, Cordon-Cardo C, Goldenberg AS, Heston WDW, Fair WR: Expression of transforming growth factor α and epidermal growth factor receptor messenger RNA in neoplastic and nonneoplastic human kidney tissue. Cancer Res 49:3407–3411, 1989.
86. Yeh YC, Tsai JF, Chuang LY, Yeh HW, Tsai JH, Florine DL, Tam JP: Elevation of transforming growth factor alpha and alpha-fetoprotein levels in patients with hepatocellular carcinoma. Cancer Res 47:896–901, 1987.
87. Arteaga CL, Hanauske AR, Clark GM, Osborne CK, Hazarika RL, Pardue F, Tio F, Von Hoff DD: Immunoreactive α transforming growth factor activity in effusions from cancer patients as a marker of tumor burden and patient prognosis. Cancer Res 48:5023–5028, 1988.
88. Lee LW, Raymond VW, Tsao M-S, Lee DC, Earp HS, Grisham JW: Clonal cosegregation of tumorigenicity with overexpression of c-*myc* and transforming growth factor α genes in chemically transformed rat liver epithelial cells. Cancer Res 51:5238–5244, 1991.
89. Valverius EM, Bates SE, Stampfer MR, Clark R, McCormick F, Salomon DS, Lippman ME, Dickson RB: Transforming growth factor α production and epidermal growth factor receptor expression in normal and oncogene transformed human mammary epithelial cells. Mol Endocrinol 3:203–214, 1989.
90. Salomon DS, Perroteau I, Kidwell WR, Tam J, Derynck R: Loss of growth responsiveness to epidermal growth factor and enhanced production of alpha-transforming growth factors in *ras*-transformed mouse mammary epithelial cells. J Cell Physiol 130:397–409, 1987.
91. Godwin AK, Lieberman MW: Early and late response to induction of *ras* T24 expression in Rat-1 cells. Oncogene 5:1231–1241, 1990.
92. Rosenthal A, Lindquist PB, Bringman TS, Goeddel DV, Derynck R: Expression in rat fibroblasts of a human transforming growth factor-α cDNA results in transformation. Cell 46:301–309, 1986.
93. Raymond VW, Lee DC, Grisham JW, Earp HS: Regulation of transforming growth factor α messenger RNA expression in a chemically transformed rat hepatic epithelial cell line by phorbol ester and hormones. Cancer Res 49:3608–3612, 1988.
94. Mueller SG, Kobrin MS, Paterson AJ, Kudlow JE: Transforming growth factor-α expression in the anterior pituitary gland: Regulation by epidermal growth factor and phorbol ester in dispersed cells. Mol Endocrinol 3:976–983, 1989.
95. Pittelkow MR, Lindquist PB, Abraham RT, Graves-Deal R, Derynck R, Coffey RJ, Jr.:

Induction of transforming growth factor-α expression in human keratinocytes by phorbol esters. J Biol Chem 264:5164–5171, 1989.
96. Kelly K, Cochran BH, Stiles CD, Leder P: Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35:603–610, 1983.
97. Kruijer W, Cooper JA, Hunter T, Verma IM: PDGF induces rapid but transient expression of the c-*fos* gene. Nature (London) 312:711–716, 1984.
98. Muller R, Bravo R, Burckhardt J, Curran T: Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. Nature (London) 312:716–720, 1984.
99. Liu SC, Sanfilippo B, Perroteau I, Derynck R, Salomon DS, Kidwell WR: Expression of transforming growth factor α (TGFα) in differentiated rat mammary tumors: Estrogen induction of TGFα production. Mol Endocrinol 1:683–692, 1987.
100. Raja RH, Paterson AJ, Shin TH, Kudlow JE: Transcriptional regulation of the human transforming growth factor-α gene. Mol Endocrinol 5:514–520, 1991.
101. Derynck R: Transforming growth factor-α: structure and biological activities. J Cell Biochem 32:293–304, 1986.
102. Bell GI, Fong NM, Stempien MM, Worsted MA, Caput D, Ku L, Urdea MS, Rall LB, Sanchez-Pescador R: Human epidermal growth factor precursor: cDNA sequence, expression *in vitro* and gene organization. Nucleic Acids Res 14:8427–8446, 1986.
103. Brissenden JE, Derynck R, Francke U: Mapping of transforming growth factor α gene on human chromosome 2 close to the breakpoint of the Burkitt's lymphoma t(2;8) variant. Cancer Res 45:5593–5597, 1985.
104. Jakobovits EB, Schlokat U, Vannice JL, Derynck R, Levinson AD: The human transforming growth factor alpha promoter directs transcription initiation from a single site in the absence of a TAT sequence. Mol Cell Biol 8:5549–5554, 1988.
105. Chen X, Azizkhan JC, Lee DC: The binding of transcription factor Sp1 to multiple sites in the rat TGFα promoter determines the level of expression and influences the site of initiation. Submitted.
106. Shankar V, Ciardiello F, Kim N, Derynck R, Liscia DS, Merlo G, Langton BC, Sheer D, Callahan R, Bassin RH, Lippman ME, Hynes N, Salomon DS: Transformation of an established mouse mammary epithelial cell line following transfection with a human transforming growth factor alpha cDNA. Mol Carcinog 2:1–11, 1989.
107. Brinster RL, Allen JM, Behringer RR, Gelinas RE, Palmiter RD: Introns increase transcriptional efficiency in transgenic mice. Proc Natl Acad Sci USA 85:836–840, 1988.
108. Sandgren EP, Luetteke NC, Palmiter RD, Brinster RL, Lee DC: Overexpression of TGFα in transgenic mice: Induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. Cell 61:1121–1135, 1990.
109. Longnecker DS: Lesions induced in rodent pancreas by azaserine and other pancreatic carcinogens. Environ Health Perspect 56:245–251, 1984.
110. Matsui Y, Halter SA, Holt JT, Hogan BLM, Coffey RJ: Development of mammary hyperplasia and neoplasia in MMTV-TGFα transgenic mice. Cell 61:1147–1155, 1990.
111. Jhappan C, Stable C, Harkins RN, Fausto N, Smith GH, Merlino GT: TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell 61:1137–1146, 1990.

12. Growth regulation by transforming growth factor-β

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Introduction

The name transforming growth factor-β (TGF-β) has come to represent a family of highly homologous polypeptides with a wide range of biological activities. The first member of this gene family was identified nearly a decade ago as one of two essential factors, called TGF-α and TGF-β present in the conditioned medium of a murine sarcoma virus-transformed cell line, which together stimulated the anchorage-independent growth of non-transformed fibroblast cell lines [1]. Several members of the TGF-β family have since been identified, of which TGF-β1, β2, and β3 are produced by mammalian cells. These three forms of TGF-β have similar biological activities in the majority of assay systems, though differences in relative potency are sometimes evident. For simplicity, we will use the name TGF-β to refer to the TGF-β family as a whole, unless otherwise specified. It should, however, be pointed out that most studies have evaluated only the biological activities of TGF-β1. Finally, a number of proteins have been identified that exhibit structural similarities to TGF-β, though with a more distant relationship than the individual TGF-β isoforms. Together with TGF-β, they constitute the TGF-β superfamily. As yet little is known about the effects of these factors on cell proliferation, and they will not be discussed here.

Following its initial identification as a stimulator of anchorage-independent growth, TGF-β was shown to be a potent regulator of cell proliferation, chemotaxis, and cell–matrix interactions. A number of excellent recent reviews are available that outline in detail our current understanding of the full scope of TGF-β's biological activities [2–4]. With respect to cell proliferation, it has now become evident that TGF-β not only induces proliferation of various cell types, but is also a potent and reversible inhibitor of cell proliferation for a wide range of cell types, including epithelial, endothelial, and hematopoietic cells. We will discuss in this chapter possible molecular mechanisms of growth regulation by TGF-β.

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TGF-β has been shown to promote cell proliferation in a handful of cell types, predominantly of mesenchymal origin. Examples include fibroblasts, osteoblasts, smooth muscle cells, and Schwann cells [5,6]. In the case of NRK-49F cells, treatment of these cells with TGF-β resulted in an increased number of cell surface receptors for epidermal growth factor (EGF) [7], suggesting that an increased sensitivity to EGF or the related TGF-α in the medium could be the basis for the TGF-β-induced increase of proliferation and the synergistic effect of TGF-β and EGF or TGF-α on anchorage independence. However, this potential mechanism of TGF-β-induced proliferation is not common to all cells that are mitogenically stimulated by TGF-β.

More insight into the mechanism of growth stimulation by TGF-β came from the observation that, in comparison with other growth factors, enhanced DNA synthesis following addition of TGF-β to cell cultures was preceded by a significantly prolonged lag period [8]. This suggested that the mitogenic effect of TGF-β for some cell types was indirect and probably due to the stimulated production of a direct mitogen acting in an autocrine manner. In this context, work from a number of investigators has focused attention on platelet-derived growth factor (PDGF). TGF-β was shown to induce c-*sis* mRNA encoding the PDGF B chain and PDGF (or PDGF-related) protein in the AKR-2B fibroblastic cell line within 4–12 hours [9]. With human foreskin fibroblasts, TGF-β has been shown to stimulate expression of the A-chain gene of PDGF. Moreover, the addition of anti-PDGF antibody to TGF-β-treated fibroblasts resulted in a 60% reduction in the magnitude of the mitogenic response [10]. The inability of anti-PDGF antibody to completely abolish TGF-β's stimulation of DNA synthesis could be due to endogenously produced PDGF interacting with its receptor intracellularly, thus resulting in an inaccessibility to the exogenously added neutralizing antibody [11]. Alternatively, elaboration of PDGF may not be the only effector mechanism for TGF-β-induced mitogenesis in these cells. It is useful to note in this regard that most cell types that are growth inhibited by TGF-β lack PDGF receptors and thus cannot establish a PDGF-based autocrine loop [12].

A similar induction of PDGF-A-chain mRNA and secretion of PDGF protein by smooth muscle cells exposed to TGF-β has been reported [13]. However, it was noticed that smooth muscle cells, as well a fibroblasts and chondrocytes, displayed a proliferative behavior with a bimodal response to TGF-β. Thus, treatment of these cells with a concentration of TGF-β 10-fold higher than that which stimulated DNA synthesis nearly abolished the mitogenic response. At these higher concentrations of TGF-β, the expression of the PDGF receptor α subunit mRNA and protein were inhibited. In this manner, a PDGF-based autocrine growth loop stimulated by low concentrations of TGF-β was blocked at the receptor level by higher con-

centrations of TGF-β [13]. Thus, at least in these cells, the effects of TGF-β on cell proliferation are a reflection of the TGF-β-modulated interaction of the PDGF-based autocrine growth regulation.

The demonstration of growth stimulation of mesenchymal cells by TGF-β under defined culture conditions can provide useful insights into possible in vivo effects of TGF-β. However, the presumed existence of concentration gradients of TGF-β in the in vivo setting poses another level of complexity for factors such as TGF-β with bimodal dose-response relationships. Exogenous TGF-β administration in vivo has been shown to result in an increased cell density, as well as fibrosis and angiogenesis [14]. However, there is as yet little in vivo evidence documenting cell proliferation in response to TGF-β. On the contrary, a recent study has revealed that an increased cell mass in the chicken chorioallantoic membrane system following administration of TGF-β was concommitant with a decreased mitogenic activity in the newly formed tissue, suggesting that the increased cell mass was largely the result of a cellular influx [15]. TGF-β is a potent chemotactic agent for a variety of cell types. Thus, many reported observations of hypercellular lesions at the site of TGF-β injection may be due to an influx of cells as a consequence of the chemotactic activity of TGF-β, rather than a TGF-β-induced increase in cell proliferation [16].

TGF-β is a potent growth inhibitor

As mentioned above, TGF-β reversibly inhibits proliferation of many cell types at picomolar concentrations. In several cases it has been shown that an anti-mitogenic effect can be achieved through an inhibition of proliferation induced by other growth stimulatory factors. In one well-studied example, TGF-β inhibited the fibroblast growth factor-stimulated proliferation of a Chinese hamster lung fibroblast cell line without affecting many of the cellular events that occur within the first few hours after mitogen stimulation. Some of the early cellular responses to mitogen that were unaffected by TGF-β included induction of ornithine decarboxylase activity, increased levels of c-*fos* mRNA, and elevated ribosomal protein S6 kinase activity [17]. Similarly, other investigators have shown that epidermal growth factor (EGF)- or insulin-stimulated proliferation of mink lung epithelial cells was inhibited by TGF-β without affecting mitogen stimulation of the ribosomal protein S6 kinase [18]. In addition, TGF-β did not inhibit the binding of growth factors to their receptors on these cells. Thus, TGF-β can inhibit the growth stimulatory effects of various mitogens without blocking specific receptor-ligand interactions or signal transduction pathways.

Specific information regarding the TGF-β receptor(s) involved in growth regulation is limited currently. It has been proposed, based on studies with TGF-β resistant mutants, that the type 1 TGF-β receptor, a glycoprotein of approximately 53 kDa, is the relevant receptor in this regard [19]. Involvement of a pertussis toxin-sensitive guanine nucleotide-binding protein (G

protein) has been suggested, based on observations with an epithelial cell line [20]. No doubt, the characterization and recent cloning of the TGF-β receptors will greatly facilitate analysis of early TGF-β-induced growth regulatory events.

Most studies on the direct inhibition of cellular proliferation by TGF-β have been performed using keratinocytes or other epithelial cells. Using synchronized cells, it has been shown that the TGF-β-induced proliferative block occurs in late G1, just prior to the onset of S phase, and thus prevents the wave of DNA synthesis [21,22]. The mechanistic nature of this inhibition is as yet largely unclear; however, some interesting functional connections have recently emerged.

Inhibition of murine keratinocyte proliferation by TGF-β was associated with diminished expression of c-*myc*, but not with a change in expression of c-*fos* or several other oncogenes. That this decrease in c-*myc* expression could be functionally involved in the inhibition of proliferation by TGF-β was suggested by an increase of c-*myc* during increased proliferation. Moreover, keratinocytes that are growth inhibited by TGF-β are inhibited to a similar extent by antisense oligonucleotides specific for c-*myc* [23]. Finally c-*myc* mRNA levels were not decreased in various tumor cell lines that are not growth inhibited by TGF-β. This is, for example, the case with keratinocytes transformed with human papillomavirus (HPV) types 16 or 18, and the simian virus 40 (SV40) [24]. An association between growth inhibition by TGF-β and decreased expression of c-*myc* has also been reported for well-differentiated colon carcinoma cells [25]. Whereas early studies attributed the observed reduction in c-*myc* by TGF-β to a post transcriptional process [26], subsequent analyses have documented a predominant inhibition of transcriptional initiation of c-*myc* by TGF-β. This inhibition of transcription was mapped to a genomic region extending from −100 to +71, relative to the 5′ most transcription initiation site of the c-*myc* gene [23]. More accurate mapping revealed the requirement of a specific 23 base pair sequence, termed the *TGF*-β control element, in this region of the c-*myc* promoter [27]. This TGF-β control element contains sequences similar to the previously described TGF-β inhibitory element (GAGTTGGTGA), which mediates the TGF-β-induced repression of transcription of the transin/stromelysin gene [28]. DNA-binding proteins specific for sequences within the TGF-β control element of the c-*myc* gene have recently been described [27].

Transfection studies with keratinocytes employing a plasmid in which chloramphenicol acetyltransferase was linked to the TGF-β repression element from the c-*myc* promoter (−100 to +71) have shown that expression of the transforming proteins from three DNA tumor viruses — E7 from HPV-16, E1A from adenovirus, and large T antigen from SV40 — could effectively block inhibition of c-*myc* transcription by TGF-β [24]. These transforming proteins are known to bind to critical growth-regulatory cellular proteins, including the retinoblastoma gene product, pRB, which has been

shown to be a tumor suppressor gene and to exert growth inhibitory activities [29]. Pietenpol et al. have further reported that a transformation-defective mutant of adenovirus E1A protein that could bind to pRB was able to prevent inhibition of c-*myc* transcription by TGF-β [24].

Thus, attention has now been focused on the possible role of pRB and related proteins as mediators of TGF-β's growth-inhibitory activity. During the cell cycle, pRB undergoes phosphorylation and dephosphorylation, with the underphosphorylated form predominating in the G1 phase [30]. Laiho et al. have shown that growth inhibition of mink lung epithelial cells by TGF-β was associated with an overall decreased phosphorylation state of pRB [21]. Futhermore, SV40 T antigen expression served to diminish the growth-inhibitory effect of TGF-β without affecting the decrease in phosphorylation of pRB [21]. Other early responses to TGF-β, such as increased expression of *jun*B mRNA, were not blocked by T antigen [31]. The hypothesis that arises from these data is that TGF-β in some manner generates or stabilizes the underphosphorylated form of pRB, which in turn mediates inhibition of cell proliferation. Transforming proteins, such as SV40 T antigen, which associate with and thereby inactivate or alter the function of the underphosphorylated form of pRB, would thus effectively block TGF-β's growth-inhibitory activity. Consistent with the hypothesis that for some cell types the growth-inhibitory effects of TGF-β require functional pRB, the human prostate carcinoma cell line DU145, which contains a mutant pRB, responds to TGF-β with a decrease in c-*myc* mRNA content but is not growth inhibited [32]. It should, however, be pointed out that it is as yet unclear whether the effect of TGF-β on the phosphorylation state of pRB is the result of a direct effect of TGF-β or a consequence of the growth arrest in G1.

In a recently published study of the ability of various mutants of the E1A transforming protein to impart resistance to growth inhibition by TGF-β in a mouse keratinocyte line, E1A-associated cellular proteins other than pRB have been implicated as well [33]. Specifically, resistance to TGF-β was greatest with an E1A mutant that retained binding to pRB as well as three other E1A binding proteins (p60, p107 and p300). When an E1A mutant capable of binding to p300, but not the other three proteins, was used, TGF-β resistance dropped to 61% of control. Thus, cellular proteins other than pRB, perhaps recessive oncogenes themselves, can also be mediators of growth inhibition by TGF-β. Such would also be the conclusion from observations that some breast cancer cell lines that lack functional pRB retain sensitivity to inhibition of proliferation by TGF-β [34]. The p53 recessive oncogene product is a candidate alternative mediator of growth inhibition by TGF-β of some cell types. For example, SV40-immortalized human bronchial epithelial cells lose their negative growth responsiveness to TGF-β when transfected with a mutant p53 [35].

The possibility remains that other late-G1-acting proteins may also be involved in TGF-β's growth-inhibitory effects. One such protein is a 34-kDa

serine-threonine kinase ($p34^{cdc2}$), which itself fluctuates in activity and phosphorylation state with the cell cycle. As with pRB, growth inhibition by TGF-β has been associated with a decrease in the level of phosphorylation of this protein [22]. Perhaps activation of a specific phosphatase by TGF-β, such as has been reported with protein phosphatase 1, is involved in these changes in the phosphorylation state of cell cycle-regulatory proteins [36].

A recent finding that pRB can itself modulate expression of the TGF-β1 gene, positively or negatively depending on cell type, adds yet another level of complexity to this growth regulatory pathway [37]. Clearly, we have much more to learn about the functional relationships between TGF-β and pRB. In a more general sense, interactions between growth-inhibitory proteins and recessive oncogene products may prove to be a fruitful area for future study.

Additional possible mechanisms for TGF-β-induced growth inhibition

Numerous investigators have reported observations that suggest mechanisms of growth inhibition by TGF-β distinct from those discussed above. TGF-β can, in some settings, downregulate the surface expression of receptors for growth-stimulatory factors. We have already reviewed the inhibition of PDGF receptor levels by TGF-β as part of a bimodal dose-response relationship in some cells stimulated to grow at low concentrations of TGF-β [13]. Similarly, TGF-β-induced decreases in high-affinity receptor densities for EGF and basic fibroblast growth factor on endothelial and osteosarcoma cells have been proposed to mediate the inhibition of proliferative response to these mitogens by TGF-β [38,39]. Inhibition of interleukin 1 receptor expression in hematopoietic cells by TGF-β may be another example of growth inhibition via a decrease in the availability of receptors for positive growth regulators [40].

Inhibition of normal rat kidney cell proliferation by TGF-β in serum-free conditions was overcome by the exogenous addition of collagenase. These cells were growth inhibited by collagen, the secretion of which was increased by TGF-β [41]. Thus, for some cells in certain settings interactions with the extracellular matrix, as modified by the actions of TGF-β, may influence cell proliferation.

Lipid peroxidation as a component of growth inhibition by TGF-β for some cells has been suggested by a report that polyunsaturated fatty acids in combination with TGF-β can result in enhanced and irreversible inhibition of cell proliferation. The antioxidant vitamin E could abolish the cytotoxicity of fatty acid plus TGF-β [42]. While it is possible that this observation represents a peculiarity of the serum-free, nonhypoxic in vitro conditions, the suggestion that cellular proliferation can be affected by oxidation events is intriguing. Further evidence supporting this hypothesis has recently been published. TGF-β has been reported to exert a prooxidant effect on bovine arterial endothelial cells, and treatment of a murine osteoblast cell line with

TGF-β stimulated the secretion of hydrogen peroxide [43,44]. Furthermore, TGF-β-induced inhibition of DNA synthesis in the osteoblastic cells was reversed by catalase [44].

As we had cautioned in the previous discussion of growth stimulation by TGF-β, in vitro experiments cannot account for the full complexity of the in vivo setting and may therefore incorrectly predict in vivo phenomena. Many of the published examples demonstrating growth inhibition by TGF-β in vivo involve localized or intravenous delivery of supraphysiologic amounts of active TGF-β. Such experimental approaches have demonstrated an inhibition of proliferation of regenerating hepatocytes, developing mouse mammary epithelia, and human tumor xenografts in athymic mice [45–47].

In this review we have focused on possible mechanisms of growth regulation by TGF-β, both positive and negative. Observations that correlate loss of responsiveness to the growth inhibitory effect of TGF-β with malignant transformation [48] underscore the importance of cellular pathways and mediators of TGF-β action as targets for carcinogenic events. A number of publications have appeared that report an altered response to TGF-β by metastatic cells compared with their nonmetastatic counterparts. Metastatic radiation- or *ras*-transformed fibroblasts were growth stimulated by TGF-β, in contrast to similarly transformed but nonmetastatic cells, which were growth inhibited by TGF-β [49]. Likewise, stimulation of soft agar colony formation by TGF-β of a metastatic melanoma cell line was abolished in cells made less metastatic by transfection with a plasmid directing synthesis of the nm23 protein [50]. These and other observations that suggest metastatic subclones may be preferentially stimulated by TGF-β [51] encourage further investigations into the molecular mechanisms that underlie the regulation of cell proliferation by TGF-β.

References

1. Anzano MA, Roberts AB, Smith JM, Sporn MB, De Larco JE: Sarcoma growth factor from conditioned medium is composed of both type alpha and type beta transforming growth factors. Proc Natl Acad Sci USA 80:6264–6268, 1983.
2. Roberts AB, Sporn MB: The transforming growth factor-βs. In: Peptide Growth Factors and their Receptors. Handbook of Experimental Pharmacology. Sporn MB, Roberts AB (ed): Springer Verlag, Heidelberg, pp 419–472, 1990.
3. Lyons RM, Moses HL: Transforming growth factors and the regulation of cell proliferation. Eur J Biochem 187:467–473, 1990.
4. Barnard JA, Lyons RM, Moses HL: The cell biology of transforming growth factor β. Biochim Biophys Acta Rev Cancer 1032:79–87, 1990.
5. Centrella M, McCarthy TL, Canalis E: Transforming growth factor β is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone. J Biol Chem 262:2869–2874, 1987.
6. Ridley AJ, Davis JB, Stroobant P, Land H: Transforming growth factors-β1 and β2 are mitogens for rat Schwann cells. J Cell Biol 109:3419–3424, 1989.
7. Assoian RK, Frolik CA, Roberts AB, Miller DM, Sporn MB: Transforming growth factor

beta controls receptor levels for epidermal growth factor in NRK fibroblasts. Cell 36:35–41, 1984.
8. Shipley GD, Tucker RF, Moses HL: Type β transforming growth factor/growth inhibitor stimulates entry of monolayer cultures of AKR-2B cells into S phase after a prolonged prereplicative interval. Proc Natl Acad Sci USA 82:4147–4151, 1985.
9. Leof EB, Proper JA, Goustin AS, Shipley GD, DiCorleto PE, Moses HL: Induction of c-*sis* mRNA and activity similar to platelet-derived growth factor by transforming growth factor β: A proposed model for indirect mitogenesis involving autocrine activity. Proc Natl Acad Sci USA 83:2453–2457, 1986.
10. Soma Y, Grotendorst GR: TGF-β stimulates primary human skin fibroblast DNA synthesis via an autocrine production of PDGF-related peptides. J Cell Physiol 140:246–253, 1989.
11. Keating MT, Williams LT: Autocrine stimulation of intracellular PDGF receptors in v-sis-transformed cells. Science 239:914–916, 1988.
12. Heldin C-H, Westermark B, Wasteson Å: Specific receptors for platelet-derived growth factor on cells derived from connective tissue and glia. Proc Natl Acad Sci USA 78:3664–3668, 1981.
13. Battegay EJ, Raines EW, Seifert RA, Bowen-Pope DF, Ross R: TGF-β induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. Cell 63:515–524, 1990.
14. Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, Fauci AS: Transforming growth factor beta: Rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci USA 83:4167–4171, 1986.
15. Yang EY, Moses HL: Transforming growth factor β1-induced changes in cell migration, proliferation, and angiogenesis in the chicken chorioallantoic membrane. J Cell Bio 111:731–741, 1990.
16. Moses HL, Yang EY, Pietenpol JA: TGF-β stimulation and inhibition of cell proliferation: New mechanistic insights. Cell 63:245–247, 1990.
17. Chambard J-C, Pouysségur J: TGF-β inhibits growth factor-induced DNA synthesis in hamster fibroblasts without affecting the early mitogenic events. J Cell Physiol 135:101–107, 1988.
18. Like B, Massagué J: The antiproliferative effect of type beta transforming growth factor occurs at a level distal from receptors for growth-activating factors. J Biol Chem 261:13426–13429, 1986.
19. Boyd FT, Massaguè J: Transforming growth factor-β inhibition of epithelial cell proliferation linked to the expressive of a 53-kDa membrane receptor. J Biol Chem 264:2272–2278, 1989.
20. Howe PH, Cunningham MR, Leof EB: Inhibition of mink lung epithelial cell proliferation by transforming growth factor-β is coupled through a pertussis-toxin-sensitive substrate. Biochem J 266:537–543, 1990.
21. Laiho M, DeCaprio JA, Ludlow JW, Livingston DM, Massagué J: Growth inhibition by TGF-β linked to suppression of retinoblastoma protein phosphorylation. Cell 62:175–185, 1990.
22. Howe PH, Draetta G, Leof EB: Transforming growth factor β1 inhibition of $p34^{cdc2}$ phosphorylation and histone H1 kinase activity is associated with G1/S phase growth arrest. Mol Cell Biol 11:1185–1194, 1991.
23. Pietenpol JA, Holt JT, Stein RW, Moses HL: Transforming growth factor β1 suppression of c-*myc* gene transcription: Role in inhibition of keratinocyte proliferation. Proc Natl Acad Sci USA 87:3758–3762, 1990.
24. Pietenpol JA, Stein RW, Moran E, Yaciuk P, Schlegel R, Lyons RM, Pittelkow MR, Münger K, Howley PM, Moses HL: TGF-β1 inhibition of c-*myc* transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. Cell 61:777–785, 1990.
25. Mulder KM, Humphrey LE, Gene Choi H, Childress-Fields KE, Brattain MG: Evidence

for c-myc in the signaling pathway for TGF-β in well-differentiated human colon carcinoma cells. J Cell Physiol 145:501–507, 1990.
26. Coffey RJ, Jr., Bascom CC, Sipes NJ, Graves-Deal R, Weissman BE, Moses HL: Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor β. Mol Cell Biol 8:3088–3093, 1988.
27. Pietenpol JA, Münger K, Howley PM, Stein RW, Moses HL: Factor-binding element in the human c-*myc* promoter involved in transcriptional regulation by transforming growth factor β1 and the retinoblastoma gene product. Proc Natl Acad Sci USA 88:10227–10231, 1991.
28. Kerr LD, Miller DB, Matrisian LM: TGF-β1 inhibition of transin/stromelysin gene expression is mediated through a fos binding sequence. Cell 61:267–278, 1990.
29. Weinberg RA: Tumor suppressor genes. Science 254:1138–1146, 1991.
30. DeCaprio JA, Ludlow JW, Lynch D, Furukawa Y, Griffin J, Piwnica-Worms H, Huang CM, Livingston DM: The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell 58:1085, 1989.
31. Laiho M, Rönnstrand L, Heino J, DeCaprio JA, Ludlow JW, Livingston DM, Massagué J: Control of junB and extracellular matrix protein expression by transforming growth factor-β1 is independent of simian virus 40 T antigen-sensitive growth-inhibitory events. Mol Cell Biol 11:972–978, 1991.
32. Zentella A, Weis FMB, Ralph DA, Laiho M, Massagué J: Early gene responses to transforming growth factor-β in cells lacking growth-suppressive RB function. Mol Cell Biol 11:4952–4958, 1991.
33. Missero C, Filvaroff E, Dotto GP: Induction of transforming growth factor β_1 resistance by the *E1A* oncogene requires binding to a specific set of cellular proteins. Proc Natl Acad Sci USA 88:3489–3493, 1991.
34. Ong G, Sikora K, Gullick WJ: Inactivation of the retinoblastoma gene does not lead to loss of TGF-β receptors or response to TGF-β in breast cancer cell lines. Oncogene 6:761–763, 1991.
35. Spillare E, Gerwin BI, Lehman TA, Forrester K, Kispert J, Pfeifer AMA, Baker SJ, Vogelstein B, Harris CC: Transforming growth factor β_1 (TGF-β_1) responsiveness and growth of human bronchial epithelial cells are affected by overexpression of exogenous wild type and mutant p53. Proc Am Assoc Cancer Res 32:308, 1991.
36. Gruppuso PA, Mikumo R, Brautigan DL, Braun L: Growth arrest induced by transforming growth factor β1 is accompanied by protein phosphatase activation in human keratinocytes. J Biol Chem 266:3444–3448, 1991.
37. Kim S-J, Lee H-D, Robbins PD, Busam K, Sporn MB, Roberts AB: Regulation of transforming growth factor β1 gene expression by the product of the retinoblastoma-susceptibility gene. Proc Natl Acad Sci USA 88:3052–3056, 1991.
38. Takehara K, LeRoy EC, Grotendorst GR: TGF-β inhibition of endothelial cell proliferation: Alteration of EGF binding and EGF-induced growth-regulatory (competence) gene expression. Cell 49:415–422, 1987.
39. Mioh H, Chen JK: Differential inhibitory effects of TGF-β on EGF-, PDGF-, and HBGF-1-stimulated MG63 human osteosarcoma cell growth: Possible involvement of growth facter interactions at the receptor and postreceptor levels. J Cell Physiol 139:509–516, 1989.
40. Dubois CM, Ruscetti FW, Palaszynski EW, Falk LA, Oppenheim JJ, Keller JR: Transforming growth factor β is a potent inhibitor of interleukin 1 (IL-1) receptor expression: Proposed mechanism of inhibition of IL-1 action. J Exp Med 172:737–744, 1990.
41. Nugent MA, Newman MJ: Inhibition of normal rat kidney cell growth by transforming growth factor-β is mediated by collagen. J Biol Chem 264:18060–18067, 1989.
42. Newman MJ: Inhibition of carcinoma and melanoma cell growth by type 1 transforming growth factor β is dependent on the presence of polyunsaturated fatty acids. Proc Natl Acad Sci USA 87:5543–5547, 1990.
43. Das SK, Fanburg BL: TGF-β_1 produces a 'prooxidant' effect on bovine pulmonary artery

endothelial cells in culture. Am J Physiol Lung Cell Mol Physiol 261:L249–L254, 1991.
44. Shibanuma M, Kuroki T, Nose K: Release of H_2O_2 and phosphorylation of 30 kilodalton proteins as early responses of cell cycle-dependent inhibition of DNA synthesis by transforming growth factor β1. Cell Growth Differ 2:583–591, 1991.
45. Russell WE, Coffey RJ, Jr, Ouellette AJ, Moses HL: Type β transforming growth factor reversibly inhibits the early proliferative response to partial hepatectomy in the rat. Proc Natl Acad Sci USA 85:5126–5130, 1988.
46. Silberstein GB, Daniel CW: Reversible inhibition of mammary gland growth by transforming growth factor-β. Science 237:291–293, 1987.
47. Twardzik DR, Ranchalis JE, McPherson JM, Ogawa Y, Gentry L, Purchio A, Plata E, Todaro GJ: Inhibition and promotion of differentiated-like phenotype of a human lung carcinoma in athymic mice by natural and recombinant forms of transforming growth factor-β. Natl Cancer Inst 81:1182–1185, 1989.
48. Manning AM, Williams AC, Game SM, Paraskeva C: Differential sensitivity of human colonic adenoma and carcinoma cells to transforming growth factor β (TGF-β): Conversion of an adenoma cell line to a tumorigenic phenotype is accompanied by a reduced response to the inhibitory effects of TGF-β. Oncogene 6:1471–1476, 1991.
49. Schwarz LC, Gingras M-C, Goldberg G, Greenberg AH, Wright JA: Loss of growth factor dependence and conversion of transforming growth factor-β_1 inhibition to stimulation in metastatic H-*ras*-transformed murine fibroblasts. Cancer Res 48:6999–7003, 1988.
50. Leone A, Flatow U, King CR, Sandeen MA, Margulies IMK, Liotta LA, Steeg PS: Reduced tumor incidence, metastatic potential, and cytokine responsiveness of *nm23*-transfected melanoma cells. Cell 65:25–35, 1991.
51. Theodorescu D, Caltabiano M, Greig R, Rieman D, Kerbel RS: Reduction of TGF-beta activity abrogates growth promoting tumor cell-cell interactions in vivo. J Cell Physiol 148:380–390, 1991.

13. Signal transduction by receptor tyrosine kinases

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Introduction

Many oncogenes exert proliferative effects on cells by influencing signal transduction pathways. Signal transduction provides a means for cells to propagate and amplify signals received from the environment to specific targets within the cell. The culmination of this pathway is DNA synthesis and cell division. Since growth is not a common event in organs of mature multicellular organisms, these pathways must be precisely regulated. The signalling process begins at the cytoplasmic membrane, where cell surface receptors interact with growth factors that are either soluble or present on other cells or in the extracellular matrix. Nontransformed cultured cells require exogenously supplied growth factors to stimulate proliferation and growth [1]. In contrast, transformed cells exhibit partial to complete relaxation of the requirements for growth factors, and factor dependence can be abrogated by the expression of oncogenes or activated forms of proto-oncogenes [1]. Oncogene products are able to overide factor dependency by mimicking the actions of ligands, their receptors, or downstream signals in the ordered procession of events that follow mitogenic stimulation [2]. Each control point in the signal transduction pathway is a potential target of deregulation by oncoproteins. Thus, an understanding of how tumorigenic events affect the cell's dependence upon growth factors requires the identification of these control points and the characterization of the interactions between the components of the signal transduction machinery.

Several growth factors and growth factor receptors that utilize tyrosine kinase activity to transmit signals have been identified as oncogenes with potential involvement in human cancers [2]. Among the growth factors with oncogenic potential is the product of the v-*sis* oncogene, first identified in the simian sarcoma virus [3,4]. v-*sis* encodes a protein highly related to the beta chain of platelet-derived growth factor (PDGF) and has been implicated as an oncogenic agent in certain astrocytomas, sarcomas, and gliomas [5,6]. v-*sis* and PDGF are potent mitogens for cultured cells of mesenchymal origin, and autocrine stimulation of PDGF receptors by expression of v-*sis* in fibroblasts results in morphological transformation

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[6]. Recent investigations into the signal transduction mechanisms used by factors such as PDGF have provided important clues as to how proliferative signals in mitogenesis and oncogenesis are propagated. The most striking finding has been the discovery that ligand-activated receptors form complexes with various intracellular proteins, many of which have potent growth-promoting activities. These proteins include proto-oncogene products and regulators of these products. This chapter will review these findings, focusing upon the primary responses of cells to PDGF. For a more detailed description of ligand–receptor tyrosine kinase interaction and of tyrosine kinase signal transduction, see Ullrich and Schlessinger [7], Cantley et al. [8], and Williams [9].

The members of the family of receptors encoding tyrosine kinase activity contain a number of common structural features important to their function. All have large glycosylated, extracellular ligand-binding domains, a single membrane-spanning region, and a cytosolic portion containing a tyrosine kinase domain [7]. The kinase domain of the PDGF receptor and several other receptor tyrosine kinases are interrupted by a region of variable length ('kinase insert'), which plays an important role in receptor signal transduction [9] (see below). The PDGF receptor functions as a homodimer or heterodimer of two related PDGF receptors (alpha and beta). Ligand addition to cells results in receptor dimerization or oligomerization, an event that alters the conformation of receptors and allows adjacent monomers to phosphorylate each other on critical tyrosine residues [7,9]. As a result of tyrosine phosphorylation and conformational change, receptors show both enhanced kinase activity towards endogenous and exogenous substrates, and an increased ability to interact with intracellular proteins.

The formation of the PDGF receptor signal transduction complex

The first evidence of the existence of complex formation between PDGF receptors and intracellular proteins was obtained from experiments that demonstrated copurification of receptors with phosphatidylinositol (PI)-3 kinase activity [10,11]. Subsequently, ligand-activated PDGF receptors were shown to copurify with six other proteins: phospholipase (PLC)-γ1, rasGTPase activating protein (rasGAP), Raf-1, $pp60^{c\text{-}src}$, $pp60^{c\text{-}yes}$, and $pp60^{c\text{-}fyn}$ [12–19] (Fig. 1). As described below, the activities of each of these proteins have been postulated to play major roles in mitogenic signal transduction. The formation of complexes between receptor tyrosine kinases and intracellular proteins may thus be an important intermediate in the transmission of growth regulatory signals. This complex has been termed a *signalling complex* or *signal transfer particle* [7,19] and is designated as a *signal transduction complex* in this chapter. Two models have been proposed as to how complex formation may facilitate signal transduction responses. In the first model, the associations between the cytoplasmic portions of

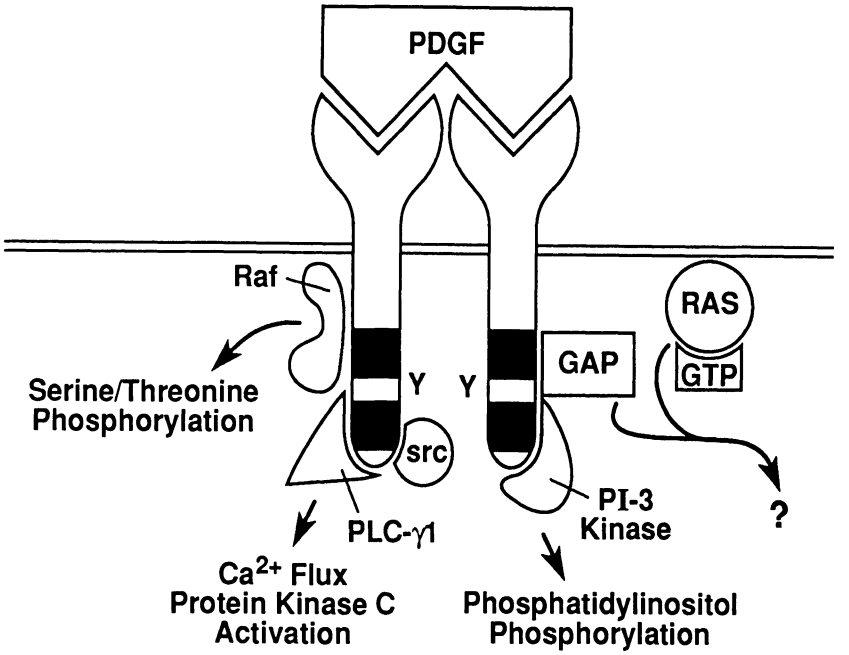

Figure 1. The PDGF receptor signal transduction complex. In cells treated with PDGF, PI-3 kinase, PLC-γ1, rasGAP (GAP), Raf-1, and $pp60^{c\text{-}src}$ (src) associate with ligand-activated PDGF receptors.

receptors and intracellular proteins may provide a mechanism for the recruitment of these proteins from the cytoplasm to the plasma membrane [8]. This would allow proteins such as PI-3 kinase and rasGAP, which are normally localized in the cytosol, to translocate to the membrane, where their substrates and targets, membrane phospholipids, and $p21^{ras}$, reside. It would also serve to colocalize signalling molecules that interact with each other. In support of this model, the levels of membrane-associated PLC-γ1, PI-3 kinase, and rasGAP increase following growth factor treatment of cells [12,20–22]. Furthermore, growth factors induce these molecules to form complexes with each other, in addition to those with receptors [16,19]. Alternatively, the associations may represent intermediates of enzyme–substrate interactions. In this model, intracellular proteins transiently interact with receptors and are then phosphorylated on tyrosine residues. Tyrosine phosphorylation of the substrate or modification of receptor interaction sites then releases the substrate protein from the receptor. Regardless of its functional significance, complex formation defines a possible pathway by which cellular proteins receive signals from ligand-activated receptors and suggests that these proteins are direct substrates of tyrosine kinases.

Further discussion of the signal transduction complex and its significance

in signalling events requires that an experimental definition of 'complex formation' be established. A common way to detect complex formation involves immunoprecipitation assays. This method involves the precipitation of receptors with specific antibodies, followed by analysis of receptor immunoprecipitates by Western blotting techniques in order to detect associated intracellular proteins. Complex formation is thus defined as protein–protein interactions that are maintained in the salt and detergent conditions used in the immunoprecipitation assays.

The associations between receptors and intracellular proteins are transient in nature and involve only a small proportion of the available receptor molecules. In PDGF-treated cells, complex formation between PDGF receptors and intracellular proteins occurs rapidly (1–5 minutes) and declines to basal levels after 60 minutes of factor treatment [10–19] (D.R. Kaplan and D.K. Morrison, unpublished). This decline in observable associations parallels the time course of receptor deactivation, which presumably occurs as a result of phosphatase activity or internalization of receptors. Thus, in cells where receptor activity cannot be downmodulated, such as PC12 cells overexpressing the trk receptor tyrosine kinase, the quantity of PL-γ1 kinase associating with the receptors does not decline for many hours (100). Only a small proportion (<10%) of PDGF receptors associate with proteins such as rasGAP, PLC-γ1, and $pp60^{c\text{-}src}$. Similar proportions of these proteins associate with receptors, indicating that the associations are either rare events or that they are extremely transient [14,16–19]. Alternatively, the immunoprecipitation assay may only detect the subpopulation of complexed proteins that are resistant to dissociation in the assay buffers containing detergent. It is likely that in intact cells a large number of intracellular proteins cycle on and off the receptors, and a small subset of these complexes are stabilized by cell lysis and are then detected in the immunoprecipitation assays.

Structural requirements for signal transduction complex formation

The association of intracellular proteins with the cytoplasmic domains of PDGF receptors occurs only when receptors are phosphorylated in response to ligand binding [11,13,15,16,19,24]. This requirement of receptor cross- (or auto-) phosphorylation for association has been demonstrated by several experimental approaches. First, removal of phosphate from ligand-activated receptors by phosphatase treatment inhibits the binding of receptors to intracellular proteins, while rephosphorylation of the dephosphorylated receptors restores binding potential [15,25]. Futhermore, mutant PDGF receptors that lack tyrosine kinase activity are incapable of associating with intracellular proteins [11,13,15,16,19]. Mutation of specific receptor phosphorylation sites also diminishes complex formation. For example, PI-3 kinase no longer associates with PDGF receptors containing mutations at the tyrosine 751 autophosphorylation site [13]. Mutation of tyrosine 857,

the most prominent receptor autophosphorylation site, prevents efficient binding of *src*-family members to PDGF receptors [24]. Strikingly, various receptor-associated proteins appear to recognize different autophosphorylation sites on the cytoplasmic domains of receptors to effect complex formation. PI-3 kinase and rasGAP recognize sites within the kinase insert region of the PDGF receptor, while PLC-γ1 and Raf-1 are capable of binding receptors in which this domain has been deleted [9,11,13,15,16,19]. Another approach to identifying receptor association sites has utilized synthetic peptides containing sequences of the autophosphorylation sites. A peptide composed of sequences of the kinase insert region, including one of the autophosphorylation sites of the receptor (tyrosine 751 of the human PDGF receptor) prevented ligand-activated receptors from associating with PI-3 kinase, but only when the peptide was phosphorylated on tyrosine residues [25]. Unphosphorylated peptide, or tyrosine-phosphorylated peptide-containing sequences unrelated to those in the kinase insert region did not inhibit association events. Thus tyrosine-phosphorylated residues in specific sequence contexts are responsible for mediating the association between PDGF receptor and PI-3 kinase.

In addition to specific receptor sequences, specific regions of intracellular proteins are required for association with receptors. One such domain identified on cellular proteins is termed *SH2* (*src* homology 2). This region is approximately 100 amino acids in length and was first identified as a domain conserved in the src family of tyrosine kinases [26]. Each of the proteins found to associate with receptors, with the exception of Raf-1, contains one or more SH2 domains. SH2 domains from rasGAP, PLC-γ1, PI-3 kinase, and $pp60^{c\text{-}src}$ bind tyrosine-phosphorylated receptors with different affinities, suggesting that it is this region that determines the binding specificity of proteins with receptors [16,19,25,27–33]. The SH2 domains are thought to be structurally similar to a pocket, where binding to negatively charged phosphotyrosine residues is stabilized by three strategically placed arginine residues (T. Pawson, personal communication).

Tyrosine phosphorylation of signal transducing proteins

Coincident with complex formation between ligand-activated receptors and cellular proteins is the phosphorylation of several of these proteins on tyrosine residues. Only unphosphorylated forms of substrates such as PLC-γ1, however, can be coimmunoprecipitated with receptors, suggesting that tyrosine phosphorylation releases the proteins from the receptor [29]. Among the receptor-associated proteins, the 85-kDa subunit of PI-3 kinase, PLC-γ1, rasGAP, Raf-1, and $pp60^{c\text{-}src}$ show increased phosphorylation on tyrosine in PDGF-treated cells [10,12,13,15–19,23,34,35]. Phosphopeptide mapping analysis of several of these proteins phosphorylated in vivo or in vitro by purified receptors indicates that the phosphorylation event is mediated directly by the activated tyrosine kinase activity of the PDGF

receptors [15,23] (D.K. Morrison, unpublished). Tyrosine phosphorylation may induce a conformational change in the substrate protein, thereby enhancing its growth-promoting ability. For two proteins, PLC-γ1 and Raf-1, tyrosine phosphorylation in vitro has been demonstrated to affect the intrinsic activity of the protein. In these experiments, the activity of these proteins was increased following phosphorylation by purified epidermal growth factor or PDGF receptors in vitro [15,36]. Other experiments demonstrate that tyrosine phosphorylation is necessary for the function of PLC-γ1 in intact cells. Mutation of PLC-γ1 at the tyrosine residue phosphorylated in vivo by PDGF receptors (tyrosine 783) completely inhibited the ability of this molecule to become activated in response to PDGF [37]. However, the mutated PLC-γ1 protein was capable of associating with PDGF receptors, indicating that the formation of complexes between PLC-γ1 and PDGF receptors is not sufficient for activation of this protein by PDGF. For the other receptor-associated proteins — rasGAP, PI-3 kinase, Raf-1, and the src family tyrosine kinases — experimental evidence is lacking as to the contribution of tyrosine phosphorylation in the modulation of the activities of these proteins.

The proteins of the signal transduction complex

The following section will provide a brief description of several proteins of the PDGF-receptor signal transduction complex. In addition to the proteins described below, immunoprecipitates of ligand-activated receptors contain several as yet unidentified proteins. The characterization of these proteins may permit a more complete understanding of PDGF signal transduction pathways. Their identification may be facilitated by the observation that SH2 domain-containing proteins associate with ligand-activated receptors. For example, three proteins containing SH2 domains, the serine/threonine kinase c-*akt*, the tyrosine phosphatase SH-PTP1, and the cellular homologue of the oncogene product v-*crk*, are prime candidates for receptor associa-

Table 1. Summary of interactions of PDGF receptor-associated proteins

	PDGF-receptor association[a]	Tyrosine phosphorylation in vivo	Activation in vivo[b]	Regulation of activity in vitro[c]
PLC-γ1	+	+	+	+
PI-3 kinase	+	+	+	?
*ras*GAP	+	+	?	?
Raf-1	+	+	+	+
Src family	+	+	+	?

[a] Protein associates with PDGF receptor in immune complexes from PDGF-treated cells.
[b] Activity of protein increases in PDGF-treated cells.
[c] Intrinsic protein activity induced by receptor tyrosine phosphorylation in in vitro assays. References in text.

tions [38–40]. Such proteins may participate in the signal transduction complex by regulating the type or extent of phosphorylation events. A summary of the interactions between intracellular proteins and the PDGF receptor is in Table 1.

PI-3 kinase

PI-3 kinase was the first protein identified that formed complexes with ligand-activated PDGF receptors [10,11]. In addition to PDGF receptors, this protein associates tightly with activated forms of virtually all receptor and nonreceptor tyrosine kinases [13,41–57]. A consensus sequence of the site on tyrosine kinases involved in PI-3 kinase interactions has been noted [8] as Y(PO_4)-MXM. This sequence is also present in IRS-1, which undergoes tyrosine phosphorylation and binds PI-3 kinase in insulin-treated cells [58]. PI-3 kinase is a cytosolic enzyme present in eukaryotic cells from yeast to mammals [59]. It catalyzes the phosphorylation of the membrane phospholipid, phosphatidylinositol, on the 3′ (D3) position of the inositol ring both in vitro and in intact cells [60,61]. The second messengers generated by PI-3 kinase activity are unknown. However, the enhancements of PI-3 activity and the association of this activity with tyrosine kinases correlate well with the ability of intracellular tyrosine kinases to transform cells or to produce tumors in vivo, and of receptor tyrosine kinases to promote mitogenesis [10,43–45,54,59,62–67]. Experimental evidence suggests that PI-3 kinase consists of at least two subunits of 85 and 110 kDa [31,68,69]. The 85-kDa subunit does not contain an ATP binding site or a kinase domain, indicating that this protein is not the catalytic subunit of PI-3 kinase [31,32,69]. However, the 85-kDa protein contains two sequences, SH2 and SH3, which are common structural elements of several cellular proteins involved in receptor tyrosine kinase signel transduction [31,32,69].

PLC-γ1

PLC-γ1 associates with several ligand-activated receptor tyrosine kinases, including the PDGF, epidermal growth factor (EGF), fibroblast growth factor (FGF), *trk*-nerve growth factor (NGF), and c-*kit* receptors [14,16, 33,54,55,70–72]. PLC-γ1 acts upon membrane phosphoinositides to produce the potent second messenger molecules: Inositol trisphosphate (IP_3), which is involved in the regulation of intracellular calcium levels, and diacylglycerol (DAG), an activator of protein kinase C [73,74]. Protein kinase C may affect many cellular processes, including gene transcription events [75] and the regulation of the proto-oncogene p21*RAS* [75]. Several experiments have addressed the requirement of PLC-γ1 activation in serum and PDGF-mediated mitogenesis. The importance of this activity was demonstrated by studies in which microinjection of antibodies to PLC-γ1 or to the PLC-γ1 substrate, PIP_2, blocked mitogenic events [77,78]. This

result is in contrast to experiments that showed that PDGF-induced DNA synthesis occurred in the absence of PLC activation [79]. The significance of PLC-γ1 in growth responses will be further elucidated by mutational analysis of receptor-PLC-γ1 association sites. For the EGF and FGF receptor tyrosine kinases, these sites have been located in the carboxy-terminal regions of the receptor [30,33].

rasGAP

rasGAP associates with the PDGF, CSF-1 receptor (c-*fms*), and c-*kit* receptors, and is phosphorylated on tyrosine residues in cells treated with PDGF, CSF-1, *Steel* factor (ligand for c-*kit*), and EGF [12,17,19,47,53,54, 80]. In addition to receptor association, growth factor treatment also induces rasGAP to complex with two cellular proteins of unknown function, p62 and p190 [22,80]. rasGAP is a negative regulator of the proto-oncogene product, $p21^{ras}$, stimulating the conversion of $p21^{ras}$ from its growth-promoting GTP-bound form to an inactive GDP-bound state [81]. One mechanism that growth factors might utilize to increase the levels of $p21^{ras}$ bound to GTP would be to inhibit rasGAP activity by tyrosine phosphorylation. Tyrosine phosphorylation, however, has no apparent effect on rasGAP activity [22]. Reductions in rasGAP activity are observed when rasGAP is complexed to p190 or to the phospholipid, phosphatidic acid, or following stimulation of the serine/threonine kinase activity of protein kinase C in T cells [22,76,82]. Increased levels of rasGAP-p190 complexes, phosphatidic acid levels, and protein kinase C activities are observed within minutes of growth factor treatment of cells, implicating these responses in rasGAP regulation. The finding that serine/threonine phosphorylation might modulate rasGAP activity is intriguing, since the serine/threonine kinase Raf-1 associates with rasGAP following PDGF treatment of cells [19]. The formation of complexes between PDGF receptors and rasGAP may also prevent rasGAP from interacting with the inhibiting the activity of $p21^{ras}$.

Raf-1

The Raf-1 serine/threonine kinase associates with ligand-activated PDGF, EGF, and c-*kit* receptors [15,53,83]. While PDGF stimulates a small increase in Raf-1 tyrosine phosphorylation (<1% of phosphate incorporated), the most evident modification is hyperphosphorylation on serine and threonine residues [84–86]. In addition, the serine/threonine kinase activity of Raf-1 is induced by treatment of cells with several growth factors that interact with receptor tyrosine kinases, including PDGF, EGF, CSF-1, and insulin [35, 87–90]. Several experiments demonstrate that Raf-1 plays a key role in mitogenesis. Microinjection of growth-arrested fibroblasts with mutant Raf-1 protein exhibiting enhanced kinase activity, induced DNA synthesis and morphological transformation [78]. In addition, expression of Raf-1

antisense oligonucleotides or kinase-defective forms of Raf-1 in fibroblasts inhibited the serum-induced proliferation of these cells [91]. Experiments using serine- or tyrosine-specific phosphatases indicate that Raf-1 activity may be regulated by both serine and tyrosine phosphorylation [90] (D.K. Morrison, unpublished). A likely substrate of Raf-1 in PDGF-treated cells may be PLC-γ1. Raf-1 and PLC-γ1 form complexes from such cells, and the sites of PLC-γ1 phosphorylated on serine in response to PDGF are similar to those phosphorylated by Raf-1 in vitro [16]. The functional consequence of this phosphorylation is not yet known.

Src-family tyrosine kinases

Three *src* family members, $pp60^{c\text{-}src}$, $pp60^{c\text{-}yes}$, and $pp60^{c\text{-}fyn}$, associate with ligand-activated PDGF receptors [18,24]. PDGF also induces a small subpopulation of cellular $pp60^{c\text{-}src}$ molecules to become activated and phosphorylated on serine and tyrosine residues [18,92,93]. The function of the src-family proteins in growth factor responses in not known. Recent experiments implicate src family proteins in the regulation of receptor tyrosine kinase activity. Expression of the oncogene, v-*src*, results in constitutive EGF receptor signalling activity and hyperphosphorylation of the receptor [94]. Thus, tyrosine phosphorylation of PDGF receptors by $pp60^{c\text{-}src}$ could enhance receptor activity, although changes in receptor phosphorylation by $pp60^{c\text{-}src}$ have not been demonstrated. $pp60^{c\text{-}src}$ may also be involved in the regulation of other members of the signal transduction complex, as $pp60^{c\text{-}src}$, together with $p21^{ras}$, are required for the activation of the kinase activity of Raf-1 in certain cells [94].

Inhibitors of signal transduction complex formation: Possible therapeutic agents

The structural and biochemical links between growth factor receptors and proto-oncogene products and their regulators support the hypothesis that subversion of growth factor pathways is one of the mechanisms involved in cell transformation. Oncogenic activation of members of the signal transduction complex may deregulate these pathways, causing constitutive proliferative signals to be transmitted. By interfering with the formation of this complex, the mitogenic activity of these proteins may be inhibited. One approach is to identify specific inhibitors of the activity of members of the complex. Two inhibitors, the actinomycete tyrphostin and the alkaloid K252, inhibit the tyrosine kinase activity of the EGF receptor and the trk (NGF) receptor, respectively, with little apparent effect on other tyrosine kinase activities [96–99]. By the use of rational drug design, selective inhibitors of cellular kinase activities may be developed. A second approach is to introduce into cells synthetic peptides that will block the associations of

kinases and their substrates [25]. This will require the identification of the interaction sites of the proteins of the signal transduction complex. The feasibility of this approach has been demonstrated by experiments in which peptides containing PDGF receptor sequences inhibited the interactions of purified PDGF receptor and PI-3 kinase in vitro.

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References

1. Cross M, Dexter TM: Growth factors in development, transformation, and tumorigenesis. Cell 64:271–280, 1991.
2. Bishop JM: Molecular themes in oncogenesis. Cell 64, 235–248, 1991.
3. Doolittle RF, Hunkapiller MW, Hood LE, Devare SG, Robbins KC, Aaronson SA, Antonidades HN: Simian sarcoma virus onc gene, v-*sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221:275–277, 1983.
4. Waterfield MD, Scrace GT, Whittle N, Stroobant P, Johnsson A, Wasteson A, Westermark B, Heldin C-H, Huang JS, Deuel TF: Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature 304:35–39, 1983.
5. Ross R, Raines EW, Bowen-Pope DF: The biology of platelet-derived growth factor. Cell 46:155–169, 1986.
6. Hannink M, Donoghue DJ: Structure and function of platelet-derived growth factor (PDGF) and related proteins. Biochim Biophys Acta 989:1–10, 1989.
7. Ullrich A, Schlessinger J: Signal transduction by receptors with tyrosine kinase activity. Cell 61:203–212, 1990.
8. Cantley LC, Auger K, Carpenter C, Duckworth B, Graziani A, Kapeller R, Solotof S: Oncogenes and signal transduction. Cell 64:281–304, 1991.
9. Williams LT: Signal transduction by the platelet-derived growth factor receptor. Science 243:1564–1570, 1989.
10. Kaplan DR, Whitman M, Schaffhausen B, Pallas DC, White M, Cantley L, Roberts TM: Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. Cell 50:1021–1029, 1987.
11. Coughlin SR, Escobedo JA, Williams LT: Role of phosphatidylinositol kinase in PDGF receptor signal transduction. Science 243:1191–1194, 1989.
12. Molloy CJ, Bottaro DP, Fleming TP, Marshall MS, Gibbs JB, Aaronson SA: PDGF induction of tyrosine phosphorylation of GTPase activating protein. Nature 342:711–714, 1989.
13. Kazalauskas A, Cooper JA: Autophosphorylation of the PDGF receptor in the kinase insert region regulates interactions with cell proteins. Cell 58:1121–1133, 1989.
14. Kumjian DA, Wahl MI, Rhee SG, Daniel TO: Platelet-derived growth factor (PDGF) binding promotes physical association of PDGF receptor with phospholipase C. Proc Natl Acad Sci USA 86:8232–8236, 1989.

15. Morrison DK, Kaplan DR, Escobedo JA, Rapp UR, Roberts TM, Williams LT: Direct activation of the serine-threonine kinase activity of Raf-1 through tyrosine phosphorylation by the PDGF β-receptor. Cell 58:649–657, 1989.
16. Morrison DK, Kaplan DR, Rhee SG, Williams LT: Platelet-derived growth factor (PDGF)-dependent association of phospholipase C-γ with the PDGF receptor signaling complex. Mol Cell Biol 10:2359–2366, 1990.
17. Kazlauskas A, Ellis C, Pawson T, Cooper JA: Binding of GAP to activated PDGF receptors. Science 247:1578–1581, 1990.
18. Kypta RM, Goldberg Y, Ulug ET, Courtneidge SA: Association between the PDGF receptor and members of the *src* family of tyrosine kinases. Cell 62:481–492, 1990.
19. Kaplan DR, Morrison DK, Wong G, McCormick F, Williams LT: PDGF β-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex. Cell 61:125–133, 1990.
20. Cohen B, Yoakim M, Piwnica-Worms H, Roberts TM, Schaffhausen BS: Tyrosine phosphorylation is a signal for the trafficking of pp85, an 85-kDa phosphorylated polypeptide associated with phosphatidylinositol kinase activity. Proc Natl Acad Sci USA 87:4458–4462, 1990.
21. Todderud G, Wahl MI, Rhee SG, Carpenter G: Stimulation of phospholipase C-g1 membrane association by epidermal growth factor. Science 249:296–298, 1990.
22. Moran MF, Polakis P, McCormick F, Pawson T, Ellis C: Protein-tyrosine kinases regulate the phosphorylation, protein interactions, subcellular distribution, and activity of P21^{ras} GTPase-activating protein. Mol Cell Biol 11:1840–1812, 1991.
23. Meisenhelder J, Suh P-G, Rhee SG, Hunter T: Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. Cell 57:1109–1122, 1989.
24. Courtneidge SA, Kypta RM, Cooper JA, Kazlauskas A: Platelet-derived growth factor receptor sequences important for binding of *src* family tyrosine kinases. Cell Growth Diff 2:483–486, 1991.
25. Escobedo JA, Kaplan DR, Kavanaugh WM, Truck CW, Williams LT: A phosphatidylinositol-3 kinase binds to platelet-derived growth factor receptor sequence containing phosphotyrosine. Mol Cell Biol 11:1124–1132, 1991.
26. Pawson T: NOn-catalytic domains of cytoplasmic protein-tyrosine kinases: Regulatory elements in signal transduction. Oncogene 3:491–495, 1988.
27. Anderson D, Koch CA, Grey L, Ellis C, Moran M, Pawson T: Binding of SH2 domains of phospholipase C-g, GAP and Src to activated growth factor receptors. Science 250: 979–982, 1990.
28. Moran MF, Koch CA, Anderson D, Ellis C, England L, Martin GS, Pawson T: Src homology region 2 domains direct protein-protein interactions in signal transduction. Proc Natl Acad Sci USA 87:8622–8626, 1990.
29. Margolis B, Bellot F, Honegger AM, Ullrich A, Schlessinger J, Zilbertstein A: Tyrosine kinase activity is essential for the association of phospholipase C-γ with the epidermal growth factor receptor. Mol Cell Biol 10:435–441, 1990.
30. Margolis B, Li N, Koch A, Mohammadi M, Hurwitz DR, Zilbertstein A, Ullrich A, Pawson T, Schlessinger J: The tyrosine phosphorylated carboxyterminus of the EGF receptor is a binding site for GAP and PLC-γ. EMBO J 9:4375–4380, 1990.
31. Escobedo JA, Navankasattusas S, Kavanaugh WM, Mifay D, Fried VA, Williams LT: Purification and cDNA cloning of novel 85 kD protein that has SH2 domains, binds to the tyrosine phosphorylated PDGF-b receptor and regulates binding of PI3-kinase to the receptor. Cell 65:75–82, 1991.
32. Skolnik JWM, Margolis B, Mohammadi M, Lowenstein E, Fisher R, Drepps A, Ullrich A, Schlessinger J: Cloning of P13-kinase associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. Cell 65:83–90, 1991.
33. Mohammadi M, Honegger AM, Rotin D, Fischer R, Bellot F, Li W, Dionne CA, Jaye M, Rubinstein, Schlessinger J: A tyrosine-phosphorylated carboxy-terminal peptide of the

fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-γ1. Mol Cell Biol 11:5068–6078, 1991.
34. Wahl MI, Olashaw NE, Nishibe S, Rhee SG, Pledger WJ, Carpenter G: Platelet-derived growth factor induces rapid and sustained tyrosine phosphorylation of phospholipase C-γ in quiescent BALB/c 3T3 cells. Mol Cell Biol 9:2934–2943, 1989.
35. Morrison DK, Kaplan DR, Rapp U, Roberts TM: Signal transduction from membrane to cytoplasm: Growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. Proc Natl Acad Sci USA 85:8855–8859, 1988.
36. Nishibe S, Wahl MI, Hernandez-Sotomayor SMT, Tonks NK, Rhee SG, Carpenter G: Increase of the catalytic activity of phospholipase C-γ1 by tyrosine phosphorylation. Science 250:1253–1256, 1990.
37. Kim HK, Kim JW, Zilbertstein A, Margolis B, Kim JG, Schlessinger J, Rhee SG: PDGF stimulation of inositol phospholipid hydrolysis requires PLC-γ phosphorylation on tyrosine residues 783 and 1254. Cell 65:435–441, 1991.
38. Mayer BJ, Hamaguchi M, Hanafusa H: A novel viral oncogene with structural similarity to phospholipase C. Nature 332:272–275, 1988.
39. Bellacosa A, Testa JR, Staal SP, Tsichlis PN: A retroviral oncogene, *akt*, encoding a serine-threonine kinase containing an SH2-like region. Science 254:274–277, 1991.
40. Plutzky J, Neel BG, Rosenberg RD: Isolation of a src homology 2-containing tyrosine phosphatase. Proc Natl Acad Sci USA 89:1123–1127, 1992.
41. Sugimoto Y, Whitman M, Cantley LC, Erikson RL: Evidence that the Rous sarcoma virus transforming gene product phosphorylates phosphatidylinositol and diacylglycerol. Proc Natl Acad Sci USA 81:2117–2121, 1984.
42. Macara IG, Marinetti GV, Balduzzi PC: Transforming protein of avian sarcoma virus UR2 is associated with phosphatidylinositol kinase activity: Possible role in tumorigenesis. Proc Natl Acad Sci USA 81:2728–2732, 1984.
43. Whitman M, Kaplan DR, Schaffhausen B, Cantley L, Roberts TM: Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. Nature 315:239–242, 1985.
44. Kaplan DR, Whitman M, Schaffhausen B, Raptis L, Garcea RL, Pallas D, Roberts TM, Cantley L: Phosphatidylinositol metabolism and polyoma-mediated transformation. Proc Natl Acad Sci USA 83:3624–3628, 1986.
45. Courtneidge SA, Heber A: An 81 kd protein complexed with middle T antigen and $pp60^{c\text{-}src}$: A possible phosphatidylinositol kinase. Cell 50:1031–1037, 1987.
46. Fukui Y, Kornbluth S, Jong SM, Wang LH, Hanafusa H: Phosphatidylinositol kinase type I activity associates with various oncogene products. Oncogene Res 4:283–292, 1989.
47. Reedijk M, Liu X, Pawson T: Interactions of phosphatidylinositol kinase, GTPase-activating protein (GAP), and GAP-associated proteins with the colony-stimulating factor 1 receptor. Mol Cell Biol 10:5601–5608, 1990.
48. Shurtleff SA, Downing JR, Rock CO, Hawkins SA, Roussel MF, Sherr CJ: Structural features of the colony-stimulating factor 1 receptor that affect its association with phosphatidylinositol 3-kinase. EMBO J 9:2415–2421, 1990.
49. Bjorge JD, Chan T-O, Antczak M, Kung H-J, Fujita DJ: Activated type 1 phosphatidylinositol kinase is associated with the epidermal growth factor (EGF) receptor following EGF stimulation. Proc Natl Acad Sci USA 87:3816–3820, 1990.
50. Endemann G, Yonezawa K, Roth RA: Phosphatidylinositol kinase or an associated protein is a substrate for the insulin receptor tyrosine kinase. J Biol Chem 265:396–400, 1990.
51. Ruderman NB, Kapeller R, White MF, Cantley LC: Activation of phosphatidylinositol 3-kinase by insulin. Proc Natl Acad Sci USA 87:1411–1415, 1990.
52. Varticovski L, Druker B, Morrison D, Cantley L, Roberts T: The colony stimulating factor-1 receptor associates with and activates phosphatidylinositol-3 kinase. Nature 342: 699–702, 1989.

53. Varticovski L, Daley G, Jackson P, Baltimore D, Cantley L: Activation of PI3-kinase in cells expressing *abl* oncogene variants. Mol Cell Biol 11, in press.
54. Herbst R, Lammers R, Schlessinger J, Ullrich A: Substrate phosphorylation specificity of the human c-*kit* receptor tyrosine kinase. J Biol Chem 266:19908–19916, 1991.
55. Rottapel R, Reedijk M, Williams DE, Lyman SD, Anderson DM, Pawson T, Bernstein A: The *Steel/W* transduction pathway: Kit autophosphorylation and its association with a unique subset of cytoplasmic signaling proteins is induced by the Steel factor. Mol Cell Biol 11:3043–3051, 1991.
56. Lev S, Givol D, Yarden Y: Interkinase domain of kit contains the binding site for phosphatidylinositol 3′ kinase. Proc Natl Acad Sci USA 89:678–682, 1992.
57. Choudhury GG, Wang L-M, Pierce J, Harvey SA, Sakaguchi AY: A mutational analysis of phosphatidylinositol-3-kinase activation by human colony-stimulating factor-1 receptor. J Biol Chem 266:8068–8072, 1991.
58. Sun XJ, Rothenberg P, Kahn CR, Backer JM, Araki E, Wilden PA, Cahill DA, Goldstein BJ, White MF: Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature 352:73–76, 1991.
59. Auger KR, Carpenter CL, Cantley LC, Varticovski L: Phosphatidylinositol 3-kinase and its novel product, phosphatidylinositol 3-phosphate, are present in *Saccharomyces cerevisiae*. J Biol Chem 264:20181–20184, 1989.
60. Whitman M, Downes CP, Keeler M, Keller T, Cantley L: Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phospatidylinositol-3 phosphate. Nature 332:644–646, 1988.
61. Auger KR, Serunian LA, Soltoff SP, Libby P, Cantley LC: PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell 57:167–175, 1989.
62. Whitman M, Kaplan D, Roberts T, Cantley L: Evidence for two distinct phosphatidylinositol kinases in fibroblasts: Implications for cellular regulation. Biochem J 247:165–174, 1987.
63. Morgan WC, Kaplan DR, Pallas DC, Roberts TM: Recombinant retroviruses that transduce middle T antigen cDNAs derived from polyomavirus mutants: Separation of focus formation and soft-agar growth in transformation assays and correlations with kinase activities in vitro. J Virol 62:3407–3414, 1988.
64. Talmage DA, Freund R, Young AT, Dahl J, Dawe CJ, Benjamin TL: Phosphorylation of middle T by pp60$^{c\text{-}src}$: A switch for binding of phosphatidylinositol 3-kinase and optimal tumorigenesis. Cell 59:55–65, 1989.
65. Fukui Y, Hanafusa H: Phosphatidylinositol kinase activity associates with viral p60^{src} protein. Mol Cell Biol 9:1651–1658, 1989.
66. Chan TO, Tanaka A, Bjorge JD, Fujita DJ: Association of type 1 phosphatidylinositol kinase activity with mutationally activated forms of human pp60$^{c\text{-}src}$. Mol Cell Biol 10:3280–3283, 1990.
67. Serunian LA, Auger KR, Roberts T, Cantley LC: Production of novel polyphosphoinositides in vivo is linked to transformation by polyoma virus middle T antigen. J Virol 64:4718–4725, 1989.
68. Carpenter CL, Duckworth BC, Auger KR, Cohen B, Schaffhausen BS, Cantley, LC: Purification and characterization of phosphoinositide 3-kinase from rat liver. J Biol Chem 265:19704–19711, 1990.
69. Otsu M, Hiles I, Gout I, Fry MJ, Ruiz-Larrea F, Panayotou G, Thompson A, Dhand R, Hsuan J, Totty N, Smith AD, Moryan, SJ, Courtneidge SA, Parker PJ, Waterfield MD: Characterization of two 85 Kd proteins that associate with receptor tyrosine kinases, middle T-pp60$^{c\text{-}src}$ complexes, and phosphatidylinositol-3 kinase. Cell 65:91–104, 1991.
70. Margolis B, Rhee SG, Felder S, Mervic M, Lyall R, Levitzki A, Ullrich A, Zilbertstein A, Schlessinger J: EGF induces tyrosine phosphorylation of phospholipase C-II: A potential mechanism for EGF receptor signaling. Cell 57:1101–1107, 1989.
71. Vetter ML, Martin-Zanca D, Parada LF, Bishop JM, Kaplan DR: NGF rapidly stimulates

tyrosine phosphorylation of phospholipase C-γ by a kinase activity associated with the product of the trk proto-oncogene. Proc Natl Acad Sci USA 88:5650–5654, 1991.
72. Burgess WH, Dionne CA, Kaplow, J, Mudd R, Friesel R, Zilbertstein A, Schlessinger J, Jaye M: Characterization and cDNA cloning of phospholipase C-γ, a major substrate for heparin binding growth factor 1 (acidic fibroblast growth factor) activated tyrosine kinase. Mol Cell Biol 10:4770–4777, 1990.
73. Whitman M, Cantley L: Phosphoinositide metabolism and the control of cell proliferation. Biochim. Biophys Acta 948:327–344, 1988.
74. Rhee SF, Suh P-G, Ryu S-H, Lee SY: Studies of inositol phospholipid-specific phospholipase C. Science 244:546–550, 1989.
75. Gilman MZ: The c-fos serum response element responds to protein kinase C-dependent and -independent signals but to cyclic AMP. Genes Dev 2:394–402, 1988.
76. Downward J, Graves JD, Warne PH, Rayter S, Cantrell DA: Stimulation of $p21^{ras}$ upon T-cell activation. Nature 346:719–723, 1990.
77. Matuoka K, Fukami K, Nakanishi O, Kawai S, Takenawa T: Mitogenesis in response to PDGF and bombesin abolished by microinjection of antibody to PIP_2. Science 239: 640–643, 1988.
78. Smith MR, Heidecker G, Rapp UR, Kung H-F: Induction of transformation and DNA synthesis after microinjection of *raf* proteins. Mol Cell Biol 10:3828–3833, 1990.
79. Hill TD, Dean NM, Mordan LJ, Lau AF, Kanemitsu MY, Boynton AL: PDGF-induced activation of phospholipase C is not required for induction of DNA synthesis. Science 248:1660–1663, 1990.
80. Ellis C, Moran M, McCormick F, Pawson T: Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. Nature 343:377–381, 1990.
81. McCormick F: *ras* GTPase activating protein: Signal transmitter and signal terminator. Cell 56:5–8, 1989.
82. Tsai M-H, Roudebush M, Dobrowolski S, Yu C-L, Gibbs JR, Stacey DW: Ras GTPase-activating protein physically associates with mitogenically active phospholipids. Mol Cell Biol 11:2785–2793, 1991.
83. App H, Hazan R, Zilbertstein A, Ullrich A, Schlessinger J, Rapp U: Epidermal growth factor (EGF) stimulates association and kinase activity of Raf-1 with the EGF receptor. Mol Cell Biol 11:913–919, 1991.
84. Morrison DK: The Raf-1 kinase as a transducer of mitogenic signals. Cancer Cells 2: 377–382, 1990.
85. Li P, Wood K, Mamon H, Haser W, Roberts T: Raf-1: A kinase currently without a cause but not lacking in effects. Cell 64:479–482, 1991.
86. Heidecker G, Kolch W, Morrison DK, Rapp UR: The role of Raf-1 phosphorylation in signal transduction. In: Vander Woude G (ed): Academic Press, 1991.
87. Baccarini M, Sabatini DM, App H, Rapp UR, Stanley ER: Colony stimulating factor-1 (CSF-1) stimulates temperature dependent phosphorylation and activation and activation of the Raf-1 proto-oncogene product. EMBO J 9:3649–3657, 1990.
88. Blackshear PJ, Haupt DM, App H, Rapp UR: Insulin activates the Raf-1 protein kinase. J Biol Chem 265:12131–12134, 1990.
89. Choudhury GG, Sylvia VL, Pfeifer A, Wang LM, Smith EA, Sakaguchi AY: Human colony stimulating factor-1 receptor activates the c-Raf-1 proto-oncogene kinase. Biochim Biophys Acta 172:154–159, 1990.
90. Kovacina KS, Yonezawa K, Brautigan DL, Tonks NK, Rapp UR, Roth RA: Insulin activates the kinase activity of the Raf-1 proto-oncogene by increasing its serine phosphorylation. J Biol Chem 265:12115–12118, 1990.
91. Kolch W, Heidecker G, Lloyd P, Rapp UR: Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. Nature 349:426–428, 1991.
92. Ralston R, Bishop JM: The product of the proto-oncogene c-*src* is modified during the

cellular response to platelet-derived growth factor. Proc Natl Acad Sci USA 82:7845–7849, 1985.
93. Gould K, Hunter T: Platelet-derived growth factor induces multisite phosphorylation of pp60c-src and increases its protein-tyrosine kinase activity. Mol Cell Biol 8:3345–3356, 1988.
94. Wasilenko WJ, Payne DM, Fitzgerald DL, Weber MJ: Phosphorylation and activation of epidermal growth factor receptors in cells transformed by the *src* oncogene. Mol Cell Biol 11:309–321, 1991.
95. Williams NG, Roberts TM, Li P: Both $p21^{ras}$ and $pp60^{v\text{-}src}$ are required, but neither alone is sufficient to activate the Raf-1 kinase. Proc Natl Acad Sci USA 89:2922–2926, 1992.
96. Yaish P, Gazit A, Gilon C, Levitzki A: Blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors. Science 242:933–935, 1988.
97. Gazit A, Yaish P, Gilon C, Levitzki A: Tyrophostins I. Synthesis and biological activity of protein tyrosine kinase inhibitors. J Med Chem 32:2344–2352, 1989.
90. Berg MM, Sternberg DW, Parada LF, Chao MV: K-252a inhibits nerve growth factor-induced *trk* proto-oncogene tyrosine phosphorylation and kinase activity. J Biol Chem 267:13–16, 1992.
99. Knusel B, Kaplan DR, Winslow JW, Rosenthal A, Burton LE, Beck KD, Rabin S, Nikolics K, Hefti F: K-252b selectively potentiates cellular actions and *trk* tyrosine phosphorylation mediated by neutotrophin-3. J Neurochem 59:715–722, 1992.
100. Hempstead BL, Rabin SJ, Kaplan L, Reid S, Parada L, Kaplan DR: Overexpression of the trk tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. Neuron, in press.

14. Involvement of G proteins, cytoplasmic calcium, phospholipases, phospholipid-derived second messengers, and protein kinases in signal transduction from mitogenic cell surface receptors

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Introduction

The binding of growth factors to cell surface receptors rapidly induces a mitogenic signal transduction cascade, which results in transcriptional activation of early response genes such as *fos* [1,2]. This mitogenic signal transduction process involves a complex series of events, including tyrosine phosphorylation, Ras G-protein-dependent effects, activation of phospholipases, and activation of protein kinase C and other cytoplasmic serine/threonine protein kinases that transduce the mitogenic signal from the cell membrane to the cell nucleus. In addition to the rapid activation of *fos* transcription, there are rapid changes in phosphorylation of the Jun protein. The newly synthesized Fos protein complexes with newly phosphorylated Jun protein via leucine zipper motifs; this produces the active Fos:Jun heterodimer known as AP-1(Fos:Jun) [3,4]. AP-1(Fos:Jun) is a transcription control factor that modulates the transcription of numerous genes that contain AP-1 control elements. The activation of AP-1(Fos:Jun) in response to mitogenic stimuli occurs during the transition between the G_0 phase (the resting stage of the cell cycle) and G_1 phase (the stage when the cell becomes committed to undergo mitosis). This initiation of the G_1 phase is followed several hours later by the activation of DNA synthesis (S phase) and eventually mitosis (M phase). This chapter focuses on the signal transduction pathways, which may connect stimulation of cell surface receptors to an important early G_1 phase response, i.e., AP-1(Fos:Jun) formation via activation of *fos* transcription and Jun phosphorylation.

The mitogenic signal transduction process is not yet well understood and several complex parallel or intersecting pathways may prove to be involved; nevertheless, a general outline of a putative signal transduction network can be postulated (Fig. 1) in which mitogenic receptor stimulation results in activation of tyrosine kinases and phospholipases, which then generate phospholipid-derived second messenger molecules; this in turn causes activation of a cytoplasmic serine/threonine protein kinase cascade that eventually results in formation of AP-1(Fos:Jun) due to activation of *fos* transcription and Jun phosphorylation.

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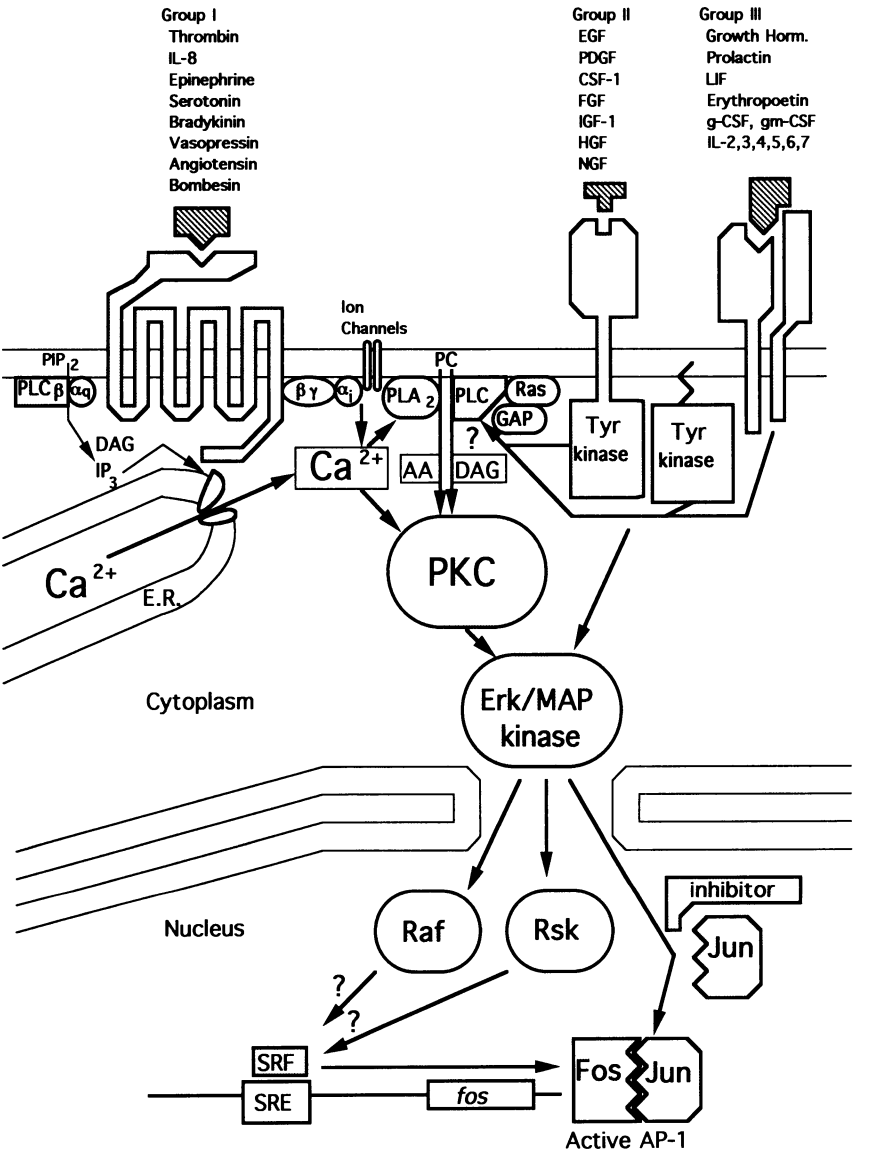

Figure 1. Schematic diagram of putative mitogenic signal transduction pathways emanating from cell surface receptors. The receptors are grouped into three categories: the seven-transmembrane-domain, trimeric-G-protein-linked, heptahelix receptor (Group I); the integral tyrosine kinase receptors (Group II); and the cytoplasmic tyrosine kinase-linked 'hematopoietin' receptors (Group III). Activation of various phospholipases causes phospholipid-derived signaling molecules (IP_3, DAG, and AA) to be generated at the plasma membrane. Subsequent events include activation of a cascade of cytoplasmic protein kinases (presumably including PKC, Erk/MAP kinase, and Raf), which eventually causes changes in phosphorylation of Jun and activation of *fos* transcription, thus leading to formation of the Fos:Jun heterodimer, which comprises the active AP-1 transcription factor. PC = phosphatidylcholine; PIP_2 = phosphatidylinositol-4,5-bisphosphate; IP_3 = inositol-1,4,5-trisphosphate; DAG = diacylglycerol; AA = arachidonic acid; PLA_2 = phospholipase A_2; PLC_β = phosphoinositide-specific phospholipase C (β isoform); $\beta\gamma,\alpha_i,\alpha_q$ = various subunits of trimeric G proteins; PLC = phosphatidylcholine-specific phospholipase C; GAP = GTPase activating protein; PKC = protein kinase C; E.R. = endoplasmic reticulum; SRF = serum response factor; SRE = serum response element.

As shown in Fig. 1 there are three major classes of mitogenic cell surface receptors. They are group I — the seven transmembrane domain-containing 'heptahelix' receptors that act through trimeric G-proteins; group II — the integral tyrosine kinase-containing growth factor receptors; and group III — the 'hematopoeitin' family of growth factor receptors that are composed of multimeric membrane-associated protein subunits that are somehow linked to rapid activation of tyrosine kinase activity.

Heptahelix receptor/trimeric (αβγ) G-protein-linked pathways

The heptahelix receptors signal via interactions with trimeric G proteins composed of α, β, and γ subunits [5,6]. An intracellular portion of the heptahelix receptor binds to GDP-bound trimeric (αβγ) G protein to produce a high-affinity for ligand conformation of the receptor. Binding of the ligand to the extracellular portion of the receptor results in dissociation of the GDP from the α-subunit and replacement by GTP; the GTP-bound α-subunit then dissociates from the βγ subunits and activates the downstream effector system [5,6]. Precisely which effector system is activated depends upon the identity of the particular heptahelix-receptor/G-protein α-subunit combination; there are several isoforms of the G-protein α-subunits, which appear to be linked to several effector systems [5,6]. For example, the α_s isoforms of the G-protein α-subunit cause activation of adenylate cyclase [5,6], whereas activation of phosphoinositide-phospholipase C-β occurs via α_q [7,8]. The α_i isoforms ($\alpha_{i\text{-}1}$, $\alpha_{i\text{-}2}$, $\alpha_{i\text{-}3}$) are thought to mediate inhibition of adenylate cylase, modulation of K^+ and Ca^{2+} channels, and activation of phospholipase A_2 [5,6,9]. Phosphoinositide-phospholipase C-β (PI-PLCβ) hydrolysis of phosphatidylinositol-4,5-bisphosphate (PI4, $5P_2$) produces inositol-1,4,5,-trisphosphate (IP_3), which releases Ca^{2+} stored within the endoplasmic reticulin, thereby elevating cytosolic Ca^{2+} [10]. Diacylglycerol (DAG), a lipid that activates protein kinase C, is the other product generated from hydrolysis of PI4,$5P_2$ by PI-PLCβ. Free arachidonic acid (AA) is produced by phospholipase A_2 (PLA_2) hydrolysis of phospholipid substrates, such as phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidylethanolamine (PE). An important function of phospholipases is to generate phospholipid-derived second messengers that activate protein kinase C (PKC). This enzyme is activated by combinations of phospholipid, Ca^{2+}, DAG, and AA [11,12]. As will be discussed in more detail below, activation of PKC causes activation of a cytoplasmic serine/threonine protein kinase cascade, which activates *fos* gene transcription and Jun phosphorylation.

Mitogenic agents that act via heptahelix G-protein-linked receptors include thrombin, bombesin, vasopressin, bradykinin, endothelin, and serotonin [13]. In fact the proto-oncogene *mas* encodes a heptahelix type receptor for angiotensin [14]. The growth associated gene *gro* encodes the

growth factor MGSA, which binds to the same receptor sites as IL-8 [15]; the IL-8 receptor is of the heptahelix type [16]. Agents that act through G-protein-linked receptors are rather weak and ineffective mitogens when acting alone, but they often show significant mitogenic actions when administered in the presence of tyrosine kinase-activating stimuli, such as insulin, EGF, or serum [13]. Mutations in the *gip2* gene (the gene that encodes $\alpha_{i\text{-}2}$) were found in some adrenal and ovarian carcinomas [17]. These mutations abrogated the GTPase activity of $\alpha_{i\text{-}2}$ by altering amino acids at positions analogous to those altered in oncogenic *ras* mutations. The benign fibroblast Rat-1 cell line could be oncogenically transformed by introduction of a mutated *gip2* gene; however, another benign fibroblast cell line (mouse 3T3) was refractory to *gip2*-induced transformation [18]. Chinese hamster ovary cells transfected with the mutated *gip2* gene showed disruption of PLA_2 stimulation by thrombin, which may represent a desensitization phenomenon associated with a constitutively activated pathway [19].

Of the various G-protein-sensitive effector systems, PI-PLCβ and PLA_2 are the two effector pathways that have received the most attention in terms of possible involvement in mitogenic activation. Recent studies have determined that α_q is the G-protein α-subunit isotype that activates PI-PLCβ [7,8]. It is unlikely that the PI-PLCβ pathway actually plays an important role in cell growth stimulation [13]. In the case of the heptahelix receptor agonist serotonin, mitogenic stimulation of smooth muscle cells and fibroblasts appears to involve an α_i subunit that is not linked to PI-PLCβ activation [20,21]. Other studies have shown that the PLA_2 pathway may be more closely linked to mitogenic activation than the PI-PLCβ pathway. In Swiss 3T3 cells, vasopressin-induced heterologous desensitization of bombesin-stimulated PLA_2 activation correlated closely with desensitization of bombesin-stimulated mitogenesis, while bombesin stimulation of PI-PLCβ did not show this correlation with desensitization of cell growth [22]. The actions of certain G-protein α-subunits ($\alpha_{i\text{-}1}$, $\alpha_{i\text{-}2}$, $\alpha_{i\text{-}3}$, and α_o but not α_q) can be blocked via ADP ribosylation by pertussis toxin [6]. An insect venom peptide called *mastoparan* can act as a molecular mimic of the intracellular portion of certain activated heptahelix receptors, thus potently activating some G-protein-mediated signal transduction pathways at a step just downstream from the heptahelix receptor [23,24]. In Swiss 3T3 cells, mastoparan stimulated both PLA_2 and cell proliferation; furthermore, both events were blocked by pertussis toxin, thus again indicating a close correlation between activation of PLA_2 and mitogenesis [25]. In Swiss 3T3 cells, lowering the extracellular Ca^{2+} concentration from 3.0 to 0.03 mM severely attenuates bombesin stimulation of PLA_2 but has no effect on bombesin stimulation of the PI-PLCβ pathway [26]. Lowering the extracellular Ca^{2+} concentration from 3.0 to 0.03 mM also resulted in an inhibition of bombesin-induced cell proliferation [27].

Recently a cytosolic PLA_2 was purified [28,29] and cloned [30,31]. This enzyme, designated c-PLA_2, is thought to represent the type of PLA_2 that

is activated by cell surface receptors because it has a similar substrate specificity (it preferentially releases arachidonic acid), and it is active at the low Ca^{2+} concentrations (10^{-8} to 10^{-6} M) that are found in the cell interior. This PLA_2 responds to Ca^{2+} by translocating from the cytoplasm to its phospholipid membrane substrate [32]; it can be further activated by polyphosphoinositide lipids and diacylglycerol [33]. This newly characterized PLA_2 contains an N-terminal domain with sequence homology to protein kinase C, GAP, and PI-PLC; this domain enables the PLA_2 to bind to its phospholipid membrane substrate at low Ca^{2+} concentrations [30].

Hydrolysis of phospholipids by phospholipase C produces DAG, a well-known activator of protein kinase C [11,12]. PKC can also be activated by arachidonic acid (AA), which is produced by PLA_2 [11,12]. Furthermore, DAG and AA act in a synergistic fashion to activate PKC [34,35]. The activation of PKC (e.g., by phorbol ester) results in a potentiation of the activation of PLA_2 by Ca^{2+} [36–38]; thus it appears that PKC can stimulate the production of its own activator (AA). Physiological mechanisms that downregulate PLA_2 activity are not well understood, but in vitro experiments with purified PLA_2 found that upon interaction with its phospholipid membrane substrate, the enzyme was rapidly autoinactivated by an unknown mechanism [39]. It is yet to be determined whether activation of PLA_2 occurs as a direct primary response to heptahelix/trimeric G-protein signaling (as in α_q activation of PI-PLCβ) or as a secondary response, resulting from PKC activation and/or changes in cytoplasmic Ca^{2+}. The α_i family of G-protein α-subunits that have been associated with PLA_2 activation have also been linked to modulation of cell membrane ion channels [9,40]. Altering ion channel activity could activate influx of Ca^{2+} across the cell membrane and thus increase cytoplasmic Ca^{2+} levels; PLA_2 activation could then occur as a secondary effect. In Madin-Darby kidney cells, elevation of cytoplasmic Ca^{2+} and activation of PKC was found to be necessary for full PLA_2 activation by G-protein-coupled receptors [38] and (as noted above) in Swiss 3T3 cells Ca^{2+} influx has been implicated in activation of PLA_2 by bombesin [27].

Initially it was assumed that the DAG produced in response to mitogenic stimulation derived entirely from PI-PLC-mediated breakdown of phosphoinositide lipids; however, it is now known that only the initial rapid peak of DAG production that occurs during the first few minutes is from PI-PLC and that the subsequent sustained elevation of DAG derives from the breakdown of phosphatidylcholine by phospholipase C and/or phospholipase D [41]. The breakdown of phosphatidylcholine by phospholipase D yields choline and phosphatidic acid. Many of the same heptahelix receptors that are linked to activation of PI-PLCβ and PLA_2 have also been linked to activation of phosphatidylcholine-phospholipase D (PC-PLD). There is evidence for involvement of both PKC and an unidentified G-protein in the activation of PC-PLD [42–46].

In Fig. 1 the heptahelix/trimeric G-protein pathway is presented as

separate from the tyrosine phosphorylation pathway; however, in some systems there appears to be some connection between these two pathways. For example, in certain systems vasopressin, epinephrine, bombesin, angiotensin, endothelin, and thrombin (which act via heptahelix receptors) can activate tyrosine phosphorylation [47–50]. In the WB rat liver epithelial cell line there is evidence for a Ca^{2+}-dependent pathway for activation of tyrosine phosphorylation by angiotensin, vasopressin, and epinephrine [47]. A possible explanation relates to the ability of some G-protein-coupled receptors to activate the pp42/Erk/MAP kinase; this enzyme is now known to exhibit kinase activity on tyrosine residues, in addition to its previously recognized serine/threonine kinase activity [51]. In a fibroblast cell line, pertussis toxin (which blocks G proteins that contain α_i subunits) inhibited thrombin-stimulated (but not FGF-stimulated) activation of the pp42/Erk/MAP kinase [52].

Tyrosine kinase/Ras G-protein linked pathways

As shown in Fig. 1, the nonheptahelix cell surface receptor mediated signalling involves tyrosine-specific protein kinases. Tyrosine kinases can be grouped into two major types: receptor and nonreceptor. The receptor tyrosine kinases are composed of an extracellular ligand-binding domain connected by a single transmembrane segment to an intracellular tyrosine kinase domain; binding of the ligand causes dimerization and activation of the tyrosine kinase domain [53]. The nonreceptor 'src family' of tyrosine kinases are associated with the cytoplamic surface of cell membranes via C-terminal myristoylation. In addition to the heptahelix and integral tyrosine kinase-containing receptor classes, there is a third class of cell growth modulating receptors, which includes the receptors for IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, g-CSF, gm-CSF, growth hormone, prolactin, LIF, and erythropoeitin [54–58]. These so-called hematopoietin receptors have a complex multimeric structure [59]. The proto-oncogene c-*mpl* encodes a member of this class of receptors [60]. Although the ligand-binding subunits do not contain intrinsic tyrosine kinase domains, many of these receptors are physically associated with tyrosine kinases [61,62] and are able to rapidly activate tyrosine phosphorylation [63–66]. The cytoplasmic portion of the IL-2β subunit of the IL-2 receptor directly associates with and activates the Lck tyrosine kinase (a src-family nonreceptor tyrosine kinase) [67,68]. The cytoplasmic portions of the hematopoietin receptors contain a region of shared sequence homology that appears to be required for transmission of growth signals [69,70]; perhaps this conserved region is involved in interaction with cytoplasmic tyrosine kinase(s).

Activated tyrosine kinases phosphorylate potential intracellular signal-tranducing substrates, including phosphoinositide phospholipase Cγ (PI-

PLCγ), the 85-kDa noncatalytic subunit of phosphoinositide 3′ kinase (PI(3)K-p85), Ras GTPase activating protein (GAP), and the 62-kDa GAP-associated tyrosine phosphoprotein (p62) [71–73]. Many of these tyrosine kinase substrates, including PI-PLCγ, GAP, and PI(3)K-p85, contain a conserved sequence motif known as the Src homology 2 (SH2) domain (this motif is also found in members of the nonreceptor src family of tyrosine kinases) [74]. The SH2 domains allow these proteins to bind in a specific fashion to certain tyrosine-phosphorylated proteins. Via their SH2 domains, PI-PLCγ, GAP, and PI(3)K-p85 are able to bind to specific tyrosine residues within the cytoplasmic domains of certain receptor tyrosine kinases when these tyrosine residues become autophosphorylated during the receptor dimerization/activation process [75,76]. The SH2 domains also enable these proteins to bind to other phosphotyrosine-containing proteins within the cell. For example, via an SH2 domain GAP binds to p62, but only when p62 is tyrosine phosphorylated [71]. It is still uncertain which of the various tyrosine kinase substrates are important in mitogenic signalling. Recent studies have indicated that PI-PLCγ and PI(3)K-p85 are probably not required for mitogenic signal transduction from receptor tyrosine kinases. The CSF-1 receptor (c-Fms) is able to potently transmit a mitogenic signal yet it does not bind to or activate PI-PLCγ [77]. Enhancement of the ability of the PDGF receptor to activate PI-PLCγ (by overexpressing PI-PLCγ) does not enhance mitogenic stimulation [78]. In the past few years a lot of interest was directed toward PI(3)K because this enzyme physically associates with (and is activated by) various activated tyrosine kinases [73,79,80]. However, more recent studies have determined that PI(3)K is probably not directly involved in mitogenic stimulation [81].

The tyrosine kinase substrate known as the GAP-associated p62 protein is particularly interesting in that it may represent a link between the two major categories of cell membrane-associated oncogene products, i.e., the tyrosine kinases and the Ras G proteins. The mitogenic signal emanating from tyrosine kinases is thought to utilize a Ras-dependent step. One of the first indications of the Ras-dependent nature of tyrosine kinase-mediated mitogenic signaling was the finding that intracellular microinjection of anti-Ras antibody abrogated the mitogenic effects of tyrosine kinase oncogenes [82]. Furthermore, there is evidence that a tyrosine kinase-mediated/Ras-dependent signaling pathway arose early in evolution. Studies involving the primitive eukaryote *C. elegans* have provided evidence for a Ras-dependent step in a receptor tyrosine kinase-mediated pathway; vulval induction requires a receptor tyrosine kinase-encoding gene (*let-23*) as well as a Ras protein-encoding gene (*let-60*) [83]. Recent work in the *Drosophila* system has also provided evidence that Ras is involved in an important signaling pathway that is activated by receptor tyrosine kinases [84,85].

The remaining sections of this chapter discuss recent evidence pointing to a pathway wherein tyrosine kinases cooperate with Ras to cause the break-

down of phosphatidylcholine and to elevate DAG, this activates protein kinase C, which leads to activation of the Erk/MAP and Raf cytoplasmic serine/threonine protein kinases.

Tyrosine kinases and Ras activate phosphatidylcholine-phospholipase C

The *Xenopus* oocyte represents a model system for signal transduction in which either receptor tyrosine kinase stimulation (via addition of insulin or IGF-1) or Ras activation (via microinjection of oncogenically activated Ras protein) can activate the oocyte meiotic maturation response as manifested by germinal vesical breakdown [86,87]. Microinjection of the oocyte with neutralizing anti-Ras antibody blocks insulin-induced oocyte maturation [87]; this is further evidence for a functional connection between the tyrosine kinase and Ras signal transduction pathways. Oocyte maturation requires activation of the maturation promoting factor (MPF), which is now known to be identical with the M-phase kinase and is composed of a complex of $p34^{cdc2}$ kinase with cyclin [88,89]. Experiments in the *Xenopus* oocyte system have shown that the addition of insulin (i.e., tyrosine kinase stimulation) or microinjection of oncogenically activated Ras protein can both cause activation of phosphatidylcholine-specific phospholipase C (PC-PLC) [90–92]. Furthermore microinjection of a purified bacterial PC-PLC enzyme mimicked the effect of insulin or microinjected Ras on oocyte maturation (germinal vesicle breakdown) and on activation of $p34^{cdc2}$ kinase [91,92]. It had previously been reported that an antibody generated against purified bacterial PC-PLC crossreacted with a mammalian PC-PLC [93]. Recently it was reported that microinjection of antibacterial PC-PLC antibody into the *Xenopus* oocyte blocked both insulin- and Ras-induced responses, including phosphocholine release (an index of PC-PLC activation), germinal vesicle breakdown, and H1-kinase ($p34^{cdc2}$ kinase/MPF) activation [91,92]. In mammalian cells there is also evidence for PC-PLC activation in response to activation of Ras [94], as well as tyrosine kinase receptor agonists such as PDGF [95,96], EGF [97,98], and CSF-1 [99,100]. Protein kinase C activation and translocation to the cell membrane was associated with the PC-PLC-induced elevation of DAG in *ras*-transformed [101] and CSF-1-stimulated [99] cells.

Activation of cytoplasmic serine/threonine kinases may transduce mitogenic signals from cell membrane to nucleus

Protein kinase C

Cells in virtually all tissues contain protein kinase C (PKC), an important regulatory enzyme that modulates exocytosis (secretion), leukocyte

superoxide generation, and cell proliferation [11,12]. The N-terminal half of PKC comprises a regulatory domain and the C-terminal half contains the protein kinase domain. There are several isozymes of PKC, designated α, β, γ, δ, ε, and ζ [11,12]. The α, β, and γ isozymes require Ca^{2+} and activating lipid (DAG and/or AA) in order to translocate from the cytoplasm to the cell membrane and become activated; these three isozymes have a C2 domain within their N-terminal halves, whereas the δ, ε, and ζ isozymes do not contain C2 domains and do not require Ca^{2+} for activation [102]. As mentioned above, GAP, PI-PLC, and c-PLA_2 also contain a C2 domain [30]. The tumor-promoting agents known as phorbol esters are able to act in place of the naturally occurring lipid activators DAG and AA, and cause direct activation of PKC [11,12]. The phorbol esters bind to a conserved motif, which is repeated twice within the regulatory domain of PKC; this region is called the C1 domain [103,104]. Interestingly, the Raf-1 protein kinase also contains a copy of this C1 motif in its regulatory domain, although neither phorbol esters nor DAG have been shown to directly activate purified Raf-1 kinase. Direct activation of protein kinase C by phorbol ester results in activation of the AP-1(Fos:Jun) transcription control factor as a result of transcriptional activation of *fos* [105] and altered phosphorylation of Jun [106]. Altering portions of the regulatory domain of PKC produces a constitutively active PKC, which causes activation of *fos* transcription in mammalian cells and activates the maturation response in the *Xenopus* oocyte system [103].

Many mitogenic stimuli (including phorbol ester activation of PKC, *ras*, and tyrosine kinase activation) cause activation of the serum response factor (SRF), a protein complex that binds to the serum response element (SRE) within the *fos* promoter [107,108]. As discussed above, tyrosine kinases and Ras may activate hydrolysis of phosphatidylcholine by PC-PLC, thus producing the PKC-activating-lipid DAG [91,94,97,101]. Introduction of activated Ras protein into mammalian cells through scrape loading caused rapid activation of PKC [109]. Specific inhibition of PKC, either by microinjection of an inhibitory peptide that mimicks the inhibitory regulatory domain of PKC or by microinjecting anti-PKC antibodies, caused blockade of Ras-induced *fos* transcription [108]. Although the mitogenic *fos*-inducing signal generated by Ras seems to be entirely PKC dependent, there is evidence that the *fos*-inducing signal generated by tyrosine kinase activation has both PKC-dependent and PKC-independent elements. PKC can be downregulated by a several hour exposure of cells to phorbol esters, which results in proteolytic degradation of the cell's PKC [110]. In PKC-downregulated cells, tyrosine kinase receptor agonists, such as PDGF and FGF, can still activate (albeit at a reduced level) *fos* transcription via the SRE, thus indicating that tyrosine kinase-mediated mitogenic stimuli can activate *fos* transcription via a PKC-independent pathway (even though these tyrosine kinases can also signal through PKC) [111]. The Erk/MAP kinase is a potentially important element of the mitogenic signal transduc-

tion pathway that can be activated by both PKC-dependent and (via tyrosine kinase-mediated stimuli) PKC-independent mechanisms, and this may account for how both PKC-dependent and PKC-independent stimuli can converge to evoke some similar downstream effects [52,112,113].

Erk/MAP kinases

The Erk/MAP kinases comprise a protein kinase family whose members include 42-kDa (Erk-2), 44-kDa (Erk-1), and 54-kDa tyrosine-phosphorylated proteins [114]. The members of this family have been called MAP2 kinases because the *m*icrotubule *a*ssociated *p*rotein-*2* is a good substrate for this kinase [112,115,116]. The terms *MAP kinase* and *Erk kinase* have also been used to denote *m*itogen *a*ctivated *p*rotein kinase [49] and *e*xtracellular signal-*r*egulated *k*inase [117]. These Erk/MAP kinases are activated by virtually all mitogenic stimuli, including tyrosine kinase receptor stimulators such as EGF, FGF, NGF, IGF-1, or insulin; activated *ras*; phorbol ester (a direct activator of PKC); and thrombin, which is an agent that acts through a heptahelix receptor/trimeric G-protein mechanism [52,114,116,118,119]. Although the Erk/MAP kinases were originally considered as tyrosine kinase substrates with serine/threonine specific kinase activity, it is now known that the Erk/MAP kinases are dual-function kinases that have intrinsic kinase activity toward tyrosine as well as serine/threonine residues [51]. The phosphorylation of tyrosine and threonine residues within the Erk/MAP kinases are both required for activation of the Erk/MAP kinases [113]; this dual phosphorylation pattern may result from phosphorylation of the Erk/MAP kinase by exogenous tyrosine and serine/threonine specific kinases as well as from autophosphorylation by the Erk/MAP kinase itself [51]. The Erk/MAP kinases can phosphorylate and activate other mitogen-activated serine/threonine protein kinases, such as Rsk/^{pp90} S6 kinase [120–123] and Raf-1 [124,125].

The Jun protein is a necessary component of the AP-1(Fos:Jun) transcription factor [3,4]. In the basal resting G_0 state, the cell's Jun protein forms an inactive complex by interacting with an inhibitor protein; the Jun protein appears to bind to this inhibitor via its A1 activation domain region [126]. The effect of activated Src or Ras is to dissociate the inhibitor protein from the Jun protein, thus allowing Jun to bind with Fos to form an active AP-1(Fos:Jun) transcription control factor [127]. This dissociation of the inhibitor protein from Jun is associated with phosphorylation of the A1 activation domain of Jun; thus the ability of activated Ras to stimulate AP-1(Fos:Jun) complex formation was found to require phosphorylation of Jun on two specific serine residues located within the A1 activation domain [128]. As discussed above, mitogenic stimuli cause activation of Erk/MAP kinase, via both PKC-dependent and PKC-independent mechanisms. When activated by mitogenic stimuli (e.g., tyrosine kinases, Ras, or phorbol ester) the Erk/MAP kinase phosphorylates the two serine residues within the A1

activation domain of Jun [118]; apparently this causes dissociation of the inhibitor protein from Jun and the resulting free phosphorylated form of Jun is able to bind to Fos and form active AP-1(Fos:Jun) factor.

Raf-1 kinase

The Raf family of serine/threonine-specific protein kinases include Raf-1 (expressed in virtually all cells), A-Raf (expressed in urogenital tissues), and B-Raf (expressed in cerebrum and testis) [129]. The structure of Raf-1 kinase shows some similarity to the structure of PKC [130]; the C-terminal half contains the catalytic kinase domain and the N-terminal half constitutes an inhibitory regulatory domain, which when absent (as in v-Raf) results in constitutively active kinase activity that can activate *fos* transcription [131, 132] and transform cells [133]. Microinjection of anti-Ras antibody blocks the mitogenic effects of cell-membrane-associated oncogene proteins, such as tyrosine kinases and Ras; however, v-Raf effects are not inhibited; this indicates that Raf acts in the mitogenic pathway downstream from tyrosine kinases and Ras [82]. A variety of growth factors, including insulin, PDGF, EGF, CSF-1, IL-2, gm-CSF, and IL-3, cause rapid serine/threonine (and in some instances, tyrosine) phosphorylation of Raf-1, and this phosphorylated form of Raf-1 is activated in terms of its kinase activity [134–137]. Growth factors and phorbol esters cause rapid activation of a serine/threonine kinase that phosphorylates and activates Raf-1 [138]. As noted above, this Raf-activating kinase has been identified as the Erk/MAP kinase [124,125]. The serum response element (SRE) is a transcription control element found in the upstream control regions of several mitogen-activated 'early response' genes, such as *fos*, β-actin, and Egr-1; v-Raf can activate the promoter of these genes [131,132,139]. A dominant inhibitory mutant of *raf*-1 (which is thought to specifically interfere with Raf-1-mediated mechanisms) blocks serum-stimulated cell proliferation and *ras*-induced cell transformation [140]. This dominant inhibitory form of Raf also blocks v-Src-induced activation of the SRE-containing Egr-1 promoter [139].

Summary

Some putative mitogenic signal transduction mechanisms involving G proteins, calcium, phospholipases, and protein kinases have been discussed. Several elements in this signal transduction scheme are not yet well understood and require further experimental investigation. With regard to the heptahelix receptors, exactly how do they activate PLA_2? Is PLA_2 activation linked to mitogenic pathways? Is this via stimulation of protein kinase C or perhaps another mechanism? How do heptahelix receptors activate tyrosine phosphorylation, and is it important in their ability to stimulate cell growth? With regard to the various phospholipases that are thought to be regulated

by receptor-mediated stimuli, only PI-PLCβ and PI-PLCγ are well characterized. PLA_2, PC-PLD, and PC-PLC require further study in regard to determination of molecular structure and elucidation of mechanisms of phospholipase activation (e.g., what are the molecular mechanisms whereby tyrosine kinases and Ras affect PC-PLC?). The protein kinase C dependent and protein kinase C independent mechanisms that enable mitogenic stimuli to activate the Erk/MAP kinase are enigmatic at this time. How Raf-1 activates SRE-containing gene promoters (such as the *fos* promoter) is also not known. However, given the current rapid rate of progress in this field, it is likely that a much more complete understanding of the mitogenic signal transduction process will soon be obtained.

References

1. Mehmet H, Rozengurt E: Regulation of c-fos expression in Swiss 3T3 cells: An interplay of multiple signal transduction pathways. Br Med Bull 47:76–86, 1991.
2. Rivera VM, Sheng M, Greenberg ME: The inner core of the serum response element mediates both the rapid induction and subsequent repression of c-fos transcription following serum stimulation. Genes Dev 4:255–268, 1990.
3. Curran T, Franza BJ: Fos and Jun: The AP-1 connection. Cell 55:395–397, 1988.
4. Verma IM, Ransone LJ, Visvader J, Sassone CP, Lamph WW: fos-jun conspiracy: Implications for the cell. Ciba Found Symp 150:128–137, 1990.
5. Bourne HR, Sanders DA, McCormick F: The GTPase superfamily: A conserved switch for diverse cell functions. Nature 348:125–132, 1990.
6. Birnbaumer L: G proteins in signal transduction. Annu Rev Pharmacol Toxicol 30: 675–705, 1990.
7. Smrcka AV, Hepler JR, Brown KO, Sternweis PC: Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. Science 251:804–807, 1991.
8. Taylor SJ, Chae HZ, Rhee SG, Exton JH: Activation of the beta 1 isozyme of phospholipase C by alpha subunits of the Gq class of G proteins. Nature 350:516–518, 1991.
9. Birnbaumer L, Abramowitz J, Yatani A, Okabe K, Mattera R, Graf R, Sanford J, Codina J, Brown AM: Roles of G proteins in coupling of receptors to ionic channels and other effector systems. Crit Rev Biochem Mol Biol 25:225–244, 1990.
10. Berridge MJ, Irvine RF: Inositol phosphates and cell signalling. Nature 341:197–205, 1989.
11. Kikkawa U, Kishimoto A, Nishizuka Y: The protein kinase C family: Heterogeneity and its implications. Annu Rev Biochem 58:31–44, 1989.
12. Nishizuka Y: The Albert Lasker Medical Awards. The family of protein kinase C for signal transduction. JAMA 262:1826–1833, 1989.
13. Moolenaar WH: G-protein-coupled receptors, phosphoinositide hydrolysis, and cell proliferation. Cell Growth Differ 2:359–364, 1991.
14. Jackson TR, Blair LA, Marshall J, Goedert M, Hanley MR: The mas oncogene encodes an angiotensin receptor. Nature 335:437–440, 1988.
15. Moser B, Schumacher C, von Tscharner V, Clark LI, Baggiolini M: Neutrophil-activating peptide 2 and gro/melanoma growth-stimulatory activity interact with neutrophil-activating peptide 1/interleukin 8 receptors on human neutrophils. J Biol Chem 266:10666–10671, 1991.
16. Thomas KM, Taylor L, Navarro J: The interleukin-8 receptor is encoded by a neutrophil-specific cDNA clone, F3R. J Biol Chem 266:14839–14841, 1991.

17. Lyons J, Landis CA, Harsh G, Vallar L, Grunewald K, Feichtinger H, Duh QY, Clark OH, Kawasaki E, Bourne HR, McCormick F: Two G protein oncogenes in human endocrine tumors. Science 249:655–659, 1990.
18. Pace AM, Wong YH, Bourne HR: A mutant alpha subunit of Gi2 induces neoplastic transformation of Rat-1 cells. Proc Natl Acad Sci USA 88:7031–7035, 1991.
19. Lowndes JM, Gupta SK, Osawa S, Johnson GL: GTPase-deficient G alpha i2 oncogene gip2 inhibits adenylylcyclase and attenuates receptor-stimulated phospholipase A2 activity. J Biol Chem 266:14193–14197, 1991.
20. Kavanaugh WM, Williams LT, Ives HE, Coughlin SR: Serotonin-induced deoxyribonucleic acid synthesis in vascular smooth muscle cells involves a novel, pertussis toxin-sensitive pathway. Mol Endocrinol 2:599–605, 1988.
21. Seuwen K, Magnaldo I, Pouyssegur J: Serotonin stimulates DNA synthesis in fibroblasts acting through 5-HT1B receptors coupled to a Gi-protein. Nature 335:254–256, 1988.
22. Millar JB, Rozengurt E: Arachidonic acid release by bombesin. A novel postreceptor target for heterologous mitogenic desensitization. J Biol Chem 265:19973–19979, 1990.
23. Higashijima T, Burnier J, Ross EM: Regulation of Gi and Go by mastoparan, related amphiphilic peptides, and hydrophobic amines. Mechanism and structural determinants of activity. J Biol Chem 265:14176–14186, 1990.
24. Weingarten R, Ransnas L, Mueller H, Sklar LA, Bokoch GM: Mastoparan interacts with the carboxyl terminus of the alpha subunit of Gi. J Biol Chem 265:11044–11049, 1990.
25. Gil J, Higgins T, Rozengurt E: Mastoparan, a novel mitogen for Swiss 3T3 cells, stimulates pertussis toxin-sensitive arachidonic acid release without inositol phosphate accumulation. J Cell Biol 113:943–950, 1991.
26. Takuwa N, Iwamoto A, Kumada M, Yamashita K, Takuwa Y: Role of Ca^{2+} influx in bombesin-induced mitogenesis in Swiss 3T3 fibroblasts. J Biol Chem 266:1403–1409, 1991.
27. Takuwa N, Kumada M, Yamashita K, Takuwa Y: Mechanisms of bombesin-induced arachidonate mobilization in Swiss 3T3 fibroblasts. J Biol Chem 266:14237–14243, 1991.
28. Clark JD, Milona N, Knopf JL: Purification of a 110-kilodalton cytosolic phospholipase A2 from the human monocytic cell line U937. Proc Natl Acad Sci USA 87:7708–7712, 1990.
29. Kramer RM, Roberts EF, Manetta J, Putnam JE: The Ca2(+)-sensitive cytosolic phospholipase A2 is a 100-kDa protein in human monoblast U937 cells. J Biol Chem 266:5268–5272, 1991.
30. Clark JD, Lin LL, Kriz RW, Ramesha CS, Sultzman LA, Lin AY, Milona N, Knopf JL: A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP. Cell 65:1043–1051, 1991.
31. Sharp JD, White DL, Chiou XG, Goodson T, Gamboa GC, McClure D, Burgett S, Hoskins J, Skatrud PL, Sportsman JR, Becker GW, Kang LH, Roberts EF, Kramer RM: Molecular cloning and expression of human Ca(2+)-sensitive cytosolic phospholipase A2. J Biol Chem 266:14850–14853, 1991.
32. Channon JY, Leslie CC: A calcium-dependent mechanism for associating a soluble arachidonoyl-hydrolyzing phospholipase A2 with membrane in the macrophage cell line RAW 264.7. J Biol Chem 265:5409–5413, 1990.
33. Leslie CC, Channon JY: Anionic phospholipids stimulate an arachidonoyl-hydrolyzing phospholipase A2 from macrophages and reduce the calcium requirement for activity. Biochim Biophys Acta 1045:261–270, 1990.
34. Lester DS, Collin C, Etcheberrigaray R, Alkon D: Arachidonic acid and diacylglycerol act synergistically to activate protein kinase C in vitro and in vivo. Biochem Biophys Res Commun 179:1522–1528, 1991.
35. Shinomura T, Asaoka Y, Oka M, Yoshida K, Nishizuka Y: Synergistic action of diacylglycerol and unsaturated fatty acid for protein kinase C activation: Its possible implications. Proc Natl Acad Sci USA 88:5149–5153, 1991.
36. Frye RA, Holz RW: Arachidonic acid release and catecholamine secretion from digitonin-treated chromaffin cells: Effects of micromolar calcium, phorbol ester, and protein

alkylating agents. J Neurochem 44:265–273, 1985.
37. Halenda SP, Banga HS, Zavoico GB, Lau LF, Feinstein MB: Synergistic release of arachidonic acid from platelets by activators of protein kinase C and Ca^{2+} ionophores. Evidence for the role of protein phosphorylation in the activation of phospholipase A2 and independence from the Na^{+}/H^{+} exchanger. Biochemistry 28:7356–7363, 1989.
38. Weiss BA, Insel PA: Intracellular Ca^{2+} and protein kinase C interact to regulate alpha 1-adrenergic- and bradykinin receptor-stimulated phospholipase A2 activation in Madin-Darby canine kidney cells. J Biol Chem 266:2126–2133, 1991.
39. Leslie CC: Kinetic properties of a high molecular mass arachidonoyl-hydrolyzing phospholipase A2 that exhibits lysophospholipase activity. J Biol Chem 266:11366–11371, 1991.
40. Brown AM, Birnbaumer L: Ionic channels and their regulation by G protein subunits. Annu Rev Physiol 52:197–213, 1990.
41. Exton JH: Signaling through phosphatidylcholine breakdown. J Biol Chem 265:1–4, 1990.
42. Martinson EA, Trilivas I, Brown JH: Rapid protein kinase C-dependent activation of phospholipase D leads to delayed 1,2-diglyceride accumulation. J Biol Chem 265:22282–22287, 1990.
43. Sandmann J, Wurtman RJ: Stimulation of phospholipase D activity in human neuroblastoma (LA-N-2) cells by activation of muscarinic acetylcholine receptors or by phorbol esters: Relationship to phosphoinositide turnover. J Neurochem 56:1312–1319, 1991.
44. Van der Meulen J, Haslam RJ: Phorbol ester treatment of intact rabbit platelets greatly enhances both the basal and guanosine 5′-[gamma-thio]triphosphate-stimulated phospholipase D activities of isolated platelet membranes. Physiological activation of phospholipase D may be secondary to activation of phospholipase C. Biochem J 271:693–700, 1990.
45. Van Blitterswijk WJ, Hilkmann H, de Widt J, van der Bend RL: Phospholipid metabolism in bradykinin-stimulated human fibroblasts. II. Phosphatidylcholine breakdown by phospholipases C and D; involvement of protein kinase C. J Biol Chem 266:10344–10350, 1991.
46. Pai JK, Pachter JA, Weinstein IB, Bishop WR: Overexpression of protein kinase C beta 1 enhances phospholipase D activity and diacylglycerol formation in phorbol ester-stimulated rat fibroblasts. Proc Natl Acad Sci USA 88:598–602, 1991.
47. Huckle WR, Prokop CA, Dy RC, Herman B, Earp S: Angiotensin II stimulates protein-tyrosine phosphorylation in a calcium-dependent manner. Mol Cell Biol 10:6290–6298, 1990.
48. Golden A, Brugge JS: Thrombin treatment induces rapid changes in tyrosine phosphorylation in platelets. Proc Natl Acad Sci USA 86:901–905, 1989.
49. Rossomando AJ, Payne DM, Weber MJ, Sturgill TW: Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. Proc Natl Acad Sci USA 86:6940–6943, 1989.
50. Zachary I, Gil J, Lehmann W, Sinnett SJ, Rozengurt E: Bombesin, vasopressin, and endothelin rapidly stimulate tyrosine phosphorylation in intact Swiss 3T3 cells. Proc Natl Acad Sci USA 88:4577–4581, 1991.
51. Seger R, Ahn NG, Boulton TG, Yancopoulos GD, Panayotatos N, Radziejewska E, Ericsson L, Bratlien RL, Cobb MH, Krebs EG: Microtubule-associated protein 2 kinases, ERK1 and ERK2, undergo autophosphorylation on both tyrosine and threonine residues: Implications for their mechanism of activation. Proc Natl Acad Sci USA 88:6142–6146, 1991.
52. L'Allemain G, Pouyssegur J, Weber MJ: p42/mitogen-activated protein kinase as a converging target for different growth factor signaling pathways: Use of pertussis toxin as a discriminating factor. Cell Regul 2:675–684, 1991.
53. Ullrich A, Schlessinger J: Signal transduction by receptors with tyrosine kinase activity. Cell 61:203–212, 1990.

54. Bazan JF: Haemopoietic receptors and helical cytokines. Immunol Today 11:350–354, 1990.
55. Bazan JF: Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci USA 87:6934–6938, 1990.
56. Bazan JF: Neuropoietic cytokines in the hematopoietic fold. Neuron 7:197–208, 1991.
57. Cosman D, Lyman SD, Idzerda RL, Beckmann MP, Park LS, Goodwin RG, March CJ: A new cytokine receptor superfamily. Trends Biochem Sci 15:265–270, 1990.
58. Gearing DP, Thut CJ, VandenBos T, Gimpel SD, Delaney PB, King J, Price V, Cosman D, Beckman MP: Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp 130. EMBO J 10:2839–2848, 1991.
59. Nicola NA, Metcalf D: Subunit promiscuity among hematopoietic growth factor receptors. Cell 67:1–4, 1991.
60. Souyri M, Vigon I, Penciolelli JF, Heard JM, Tambourin P, Wendling F: A putative truncated cytokine receptor gene transduced by the myeloproliferative leukemia virus immortalizes hematopoietic progenitors. Cell 63:1137–1147, 1990.
61. Carter-Su C, Stubbart JR, Wang XY, Stred SE, Argetsinger LS, Shafer JA: Phosphorylation of highly purified growth hormone receptors by a growth hormone receptor-associated tyrosine kinase. J Biol Chem 264:18654–18661, 1989.
62. Fung MR, Scearce RM, Hoffman JA, Peffer NJ, Hammes SR, Hosking JB, Schmandt R, Kuziel WA, Haynes BF, Mills GB, Greene WC: A tyrosine kinase physically associates with the beta-subunit of the human IL-2 receptor. J Immunol 147:1253–1260, 1991.
63. Murata Y, Yamaguchi N, Hitoshi Y, Tominaga A, Takatsu K: Interleukin 5 and interleukin 3 induce serine and tyrosine phosphorylations of several cellular proteins in an interleukin 5-dependent cell line. Biochem Biophys Res Commun 173:1102–1108, 1990.
64. Kanakura Y, Druker B, Cannistra SA, Furukawa Y, Torimoto Y, Griffin JD: Signal transduction of the human granulocyte-macrophage colony-stimulating factor and interleukin-3 receptors involves tyrosine phosphorylation of a common set of cytoplasmic proteins. Blood 76:706–715, 1990.
65. Isfort RJ, Ihle JN: Multiple hematopoietic growth factors signal through tyrosine phosphorylation. Growth Factors 2:213–220, 1990.
66. Quelle FW, Wojchowski DM: Proliferative action of erythropoietin is associated with rapid protein tyrosine phosphorylation in responsive B6SUt.EP cells. J Biol Chem 266:609–614, 1991.
67. Hatakeyama M, Kono T, Kobayashi N, Kawahara A, Levin SD, Perlmutter RM, Taniguchi T: Interaction of the IL-2 receptor with the src-family kinase p56lck: Identification of novel intermolecular association. Science 252:1523–1528, 1991.
68. Horak ID, Gress RE, Lucas PJ, Horak EM, Waldmann TA, Bolen JB: T-lymphocyte interleukin 2-dependent tyrosine protein kinase signal transduction involves the activation of p56lck. Proc Natl Acad Sci USA 88:1996–2000, 1991.
69. Fukunaga R, Ishizaka-Ikeda E, Pan C-X, Seto Y, Nagata S: Functional domains of the granulocyte colony-stimulating factor receptor. EMBO J 10:2855–2865, 1991.
70. Murakami M, Narazaki M, Hibi M, Yawata H, Yasukawa K, Hamaguchi M, Taga T, Kishimoto T: Critical cytoplasmic region of the interleukin 6 signal transducer gp 130 is conserved in the cytokine receptor family. Proc Natl Acad Sci USA 88:11349–11353, 1991.
71. Moran MF, Koch CA, Anderson D, Ellis C, England L, Martin GS, Pawson T: Src homology region 2 domains direct protein-protein interactions in signal transduction. Proc Natl Acad Sci USA 87:8622–8626, 1990.
72. Hunter T: Cooperation between oncogenes. Cell 64:249–270, 1991.
73. Cantley LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R, Soltoff S: Oncogenes and signal transduction. Cell 64:281–302, 1991.
74. Koch CA, Anderson D, Moran MF, Ellis C, Pawson T: SH2 and SH3 domains: Elements that control interactions of cytoplasmic signaling proteins. Science 252:668–674, 1991.
75. Margolis B, Li N, Koch A, Mohammadi M, Hurwitz DR, Zilberstein A, Ullrich A,

Pawson T, Schlessinger J: The tyrosine phosphorylated carboxyterminus of the EGF receptor is a binding site for GAP and PLC-gamma. Embo J 9:4375–4380, 1990.
76. Anderson D, Koch CA, Grey L, Ellis C, Moran MF, Pawson T: Binding of SH2 domains of phospholipase C gamma 1, GAP, and Src to activated growth factor receptors. Science 250:979–982, 1990.
77. Hartmann T, Seuwen K, Roussel MF, Sherr CJ, Pouyssegur J: Functional expression of the human receptor for colony-stimulating factor 1 (CSF-1) in hamster fibroblasts: CSF-1 stimulates Na^+/H^+ exchange and DNA-synthesis in the absence of phosphoinositide breakdown. Growth Factors 2:289–300, 1990.
78. Margolis B, Zilberstein A, Franks C, Felder S, Kremer S, Ullrich A, Rhee SG, Skorecki K, Schlessinger J: Effect of phospholipase C-gamma overexpression on PDGF-induced second messengers and mitogenesis. Science 248:607–610, 1990.
79. Kaplan DR, Whitman M, Schaffhausen B, Pallas DC, White M, Cantley L, Roberts TM: Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. Cell 50:1021–1029, 1987.
80. Coughlin SR, Escobedo JA, Williams LT: Role of phosphatidylinositol kinase in PDGF receptor signal transduction. Science 243:1191–1194, 1989.
81. Yu JC, Heidaran MA, Pierce JH, Gutkind JS, Lombardi D, Ruggiero M, Aaronson SA: Tyrosine mutations within the alpha platelet-derived growth factor receptor kinase insert domain abrogate receptor-associated phosphatidylinositol-3 kinase activity without affecting mitogenic or chemotactic signal transduction. Mol Cell Biol 11:3780–3785, 1991.
82. Smith MR, DeGudicibus SJ, Stacey DW: Requirement for c-ras proteins during viral oncogene transformation. Nature 320:540–543, 1986.
83. Aroian RV, Koga M, Mendel JE, Ohshima Y, Sternberg PW: The let-23 gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. Nature 348:693–699, 1990.
84. Simon MA, Bowtell DDL, Dodson GS, Laverty TR, Rubin GM: Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. Cell 67:701–716, 1991.
85. Fortini ME, Simon MA, Rubin GM: Signalling by the sevenless protein tyrosine kinase is mimicked by Ras1 activation. Nature 355:559–561, 1992.
86. Birchmeier C, Broek D, Wigler M: ras proteins can induce meiosis in Xenopus oocytes. Cell 43:615–621, 1985.
87. Korn LJ, Siebel CW, McCormick F, Roth RA: Ras p21 as a potential mediator of insulin action in Xenopus oocytes. Science 236:840–843, 1987.
88. Gautier J, Norbury C, Lohka M, Nurse P, Maller J: Purified maturation-promoting factor contains the product of a Xenopus homolog of the fission yeast cell cycle control gene cdc2+. Cell 54:433–439, 1988.
89. Erikson E, Maller JL: Biochemical characterization of the p34cdc2 protein kinase component of purified maturation-promoting factor from Xenopus eggs. J Biol Chem 264:19577–19582, 1989.
90. Lacal JC: Diacylglycerol production in *Xenopus laevis* oocytes after microinjection of p21ras proteins is a consequence of activation of phosphatidylcholine metabolism. Mol Cell Biol 10:333–340, 1990.
91. Garcia de Herreros A, Dominguez I, Diaz-Meco MT, Graziani G, Cornett ME, Guddal PH, Johansen T, Moscat J: Requirement of phospholipase C-catalyzed hydrolysis of phosphatidylcholine for maturation of *Xenopus laevis* oocytes in response to insulin and ras p21. J Biol Chem 266:6825–6829, 1991.
92. Dominguez I, Marshall MS, Gibbs JB, Garcia de Herreros A, Cornet ME, Graziani G, Diaz-Meco MT, Johansen T, McCormick F, Moscat J: Role of GTPase activating protein in mitogenic signalling through phosphatidylcholine-hydrolysing phospholipase C. EMBO J. 10:3215–3220, 1991.
93. Clark MA, Shorr RG, Bomalaski JS: Antibodies prepared to *Bacillus cereus*

phospholipase C crossreact with a phosphatidylcholine preferring phospholipase C in mammalian cells. Biochem Biophys Res Commun 140:114–119, 1986.
94. Lopez-Barahona M, Kaplan PL. Cornet ME, Diaz-Meco MT, Larrodera P, Diaz-Laviada I, Municio AM, Moscat J: Kinetic evidence of a rapid activation of phosphatidylcholine hydrolysis by Ki-ras oncogene. Possible involvement in late steps of the mitogenic cascade. J Biol Chem 265:9022–9026, 1990.
95. Besterman JM, Duronio V, Cuatrecasas P: Rapid formation of diacylglycerol from phosphatidylcholine: A pathway for generation of a second messenger. Proc Natl Acad Sci USA 83:6785–6789, 1986.
96. Larrodera P, Cornet ME, Diaz-Meco MT, Lopez-Barahona M, Diaz-Laviada I, Guddal PH, Johansen T, Moscat J: Phospholipase C-mediated hydrolysis of phosphatidylcholine is an important step in PDGF-stimulated DNA synthesis. Cell 61:1113–1120, 1990.
97. Wright TM, Shin HS, Raben DM: Sustained increase in 1,2-diacylglycerol precedes DNA synthesis in epidermal-growth-factor-stimulated fibroblasts. Evidence for stimulated phosphatidylcholine hydrolysis. Biochem J 267:501–507, 1990.
98. Fisher GJ, Henderson PA, Voorhees JJ, Baldassare JJ: Epidermal growth factor-induced hydrolysis of phosphatidylcholine by phospholipase D and phospholipase C in human dermal fibroblasts. J Cell Physiol 146:309–317, 1991.
99. Imamura K, Dianoux A, Nakamura T, Kufe D: Colony-stimulating factor 1 activates protein kinase C in human monocytes. EMBO J 9:2423–2428, 1990.
100. Choudhury GG, Sylvia VL, Sakaguchi AY: Activation of a phosphatidylcholine-specific phospholipase C by colony stimulating factor 1 receptor requires tyrosine phosphorylation and a guanine nucleotide-binding protein. J Biol Chem 266:23147–23151, 1991.
101. Diaz LI, Larrodera P, Diaz MM, Cornet ME, Guddal PH, Johansen T, Moscat J: Evidence for a role of phosphatidylcholine-hydrolysing phospholipase C in the regulation of protein kinase C by ras and src oncogenes. EMBO J 9:3907–3912, 1990.
102. Ryves WJ, Evans AT, Olivier AR, Parket PJ, Evans FJ: Activation of the PKC-isotypes alpha, $beta_1$, gamma, delta and epsilon by phorbol esters of different biological activites. FEBS Lett 288:5–9, 1991.
103. Kaibuchi K, Fukumoto Y, Oku N, Takai Y, Arai K, Muramatsu M: Molecular genetic analysis of the regulatory and catalytic domains of protein kinase C. J Biol Chem 264:13489–13496, 1989.
104. Ono Y, Fujii T, Igarashi K, Kuno T, Tanaka C, Kikkawa U, Nishizuka Y: Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence. Proc Natl Acad Sci USA 86:4868–4871, 1989.
105. Fisch TM, Prywes R, Roeder RG: c-fos sequence necessary for basal expression and induction by epidermal growth factor, 12-O-tetradecanoyl phorbol-13-acetate and the calcium ionophore. Mol Cell Biol 7:3490–3502, 1987.
106. Boyle WJ, Smeal T, Defize LH, Angel P, Woodgett JR, Karin M, Hunter T: Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. Cell 64:573–584, 1991.
107. Gilman MZ: The c-fos serum response element responds to protein kinase C-dependent and -independent signals but not to cyclic AMP. Genes Dev 2:394–402, 1988.
108. Gauthier RC, Fernandez A, Lamb NJ: ras-induced c-fos expression and proliferation in living rat fibroblasts involves C-kinase activation and the serum response element pathway. Embo J 9:171–180, 1990.
109. Morris JD, Price B, Lloyd AC, Self AJ, Marshall CJ, Hall A: Scrape-loading of Swiss 3T3 cells with ras protein rapidly activates protein kinase C in the absence of phosphoinositide hydrolysis. Oncogene 4:27–31, 1989.
110. Rodriguez PA, Rozengurt E: Disappearance of Ca^{2+}-sensitive, phospholipid-dependent protein kinase activity in phorbol ester-treated 3T3 cells. Biochem Biophys Res Commun 120:1053–1059, 1984.
111. Stumpo DJ, Blackshear PJ: Insulin and growth factor effects on c-fos expression in normal

and protein kinase C-deficient 3T3-L1 fibroblasts and adipocytes. Proc Natl Acad Sci USA 83:9453–9457, 1986.
112. Hoshi M, Nishida E, Sakai H: Characterization of a mitogen-activated, Ca^{2+}-sensitive microtubule-associated protein-2 kinase. Eur J Biochem 184:477–486, 1989.
113. Anderson NG, Maller JL, Tonks NK, Sturgill TW: Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. Nature 343:651–653, 1990.
114. Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD: ERKs: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 65:663–675, 1991.
115. Ray LB, Sturgill TW: Rapid stimulation by insulin of a serine/threonine kinase in 3T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 in vitro. Proc Natl Acad Sci USA 84:1502–1506, 1987.
116. Hoshi M, Nishida E, Sakai H: Activation of a Ca^{2+}-inhibitable protein kinase that phosphorylates microtubule-associated protein 2 in vitro by growth factors, phorbol esters, and serum in quiescent cultured human fibroblasts. J Biol Chem 263:5396–5401, 1988.
117. Boulton TG, Gregory JS, Cobb MH: Purification and properties of extracellular signal-regulated kinase 1, an insulin-stimulated microtubule-associated protein 2 kinase. Biochemistry 30:278–286, 1991.
118. Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E, Woodgett JR: Phosphorylation of c-jun mediated by MAP kinases. Nature 353:670–674, 1991.
119. Leevers SJ, Marshall CJ: Activation of extracellular signal-regulated kinase, ERK 2, by p21ras oncoprotein. EMBO J 11:569–574, 1992.
120. Sturgill TW, Ray LB, Erikson E, Maller JL: Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. Nature 334:715–718, 1988.
121. Chung J, Chen RH, Blenis J: Coordinate regulation of pp90rsk and a distinct protein-serine/threonine kinase activity that phosphorylates recombinant pp90rsk in vitro. Mol Cell Biol 11:1868–1874, 1991.
122. Chung J, Pelech SL, Blenis J: Mitogen-activated Swiss mouse 3T3 RSK kinases I and II are related to pp44mpk from sea star oocytes and participate in the regulation of pp90rsk activity. Proc Natl Acad Sci USA 88:4981–4985, 1991.
123. Chen RH, Chung J, Blenis J: Regulation of pp90rsk phosphorylation and S6 phosphotransferase activity in Swiss 3T3 cells by growth factor-, phorbol ester-, and cyclic AMP-mediated signal transduction. Mol Cell Biol 11:1861–1867, 1991.
124. Anderson NG, Li P, Marsden LA, Williams N, Roberts TM, Sturgill TW: Raf-1 is a potential substrate for mitogen-activated protein kinase in vivo. Biochem J 277:573–576, 1991.
125. Lee R-M, Cobb MH, Blackshear PJ: Evidence that extracellular signal-regulated kinases are the insulin-activated Raf-1 kinase kinases. J Biol Chem 267:1088–1092, 1992.
126. Baichwal VR, Tjian R: Control of c-Jun activity by interaction of a cell-specific inhibitor with regulatory domain delta: Differences between v- and c-Jun. Cell 63:815–825, 1990.
127. Baichwal VR, Park A, Tjian R: v-Src and EJ Ras alleviate repression of c-Jun by a cell-specific inhibitor. Nature 352:165–168, 1991.
128. Smeal T, Binetruy B, Mercola DA, Birrer M, Karin M: Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. Nature 354:494–496, 1991.
129. Rapp UR: Role of Raf-1 serine/threonine protein kinase in growth factor signal transduction. Oncogene 6:495–500, 1991.
130. Beck TW, Huleihel M, Gunnell M, Bonner TI, Rapp UR: The complete coding sequence of the human A-raf-1 oncogene and transforming activity of a human A-raf carrying retrovirus. Nucleic Acids Res 15:595–609, 1987.
131. Kaibuchi K, Fukumoto Y, Oku N, Hori Y, Yamamoto T, Toyoshima K, Takai Y: Activation of the serum response element and 12-O-tetradecanoylphorbol-13-acetate

response element by the activated c-raf-1 protein in a manner independent of protein kinase C. J Biol Chem 264:20855–20858, 1989.
132. Jamal S, Ziff E: Transactivation of c-fos and beta-actin genes by raf as a step in early response to transmembrane signals. Nature 344:463–466, 1990.
133. Heidecker G, Huleihel M, Cleveland JL, Kolch W, Beck TW, Lloyd P, Pawson T, Rapp UR: Mutational activation of c-raf-1 and definition of the minimal transforming sequence. Mol Cell Biol 10:2503–2512, 1990.
134. Morrison DK, Kaplan DR, Rapp U, Roberts TM: Signal transduction from membrane to cytoplasm: Growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. Proc Natl Acad Sci USA 85:8855–8859, 1988.
135. Carroll MP, Clark LI, Rapp UR, May WS: Interleukin-3 and granulocyte-macrophage colony-stimulating factor mediate rapid phosphorylation and activation of cytosolic c-raf. J Biol Chem 265:19812–19817, 1990.
136. Carroll MP, Spivak JL, McMahon M, Weich N, Rapp UR, May WS: Erythropoietin induces Raf-1 activation and Raf-1 is required for erythropoietin-mediated proliferation. J Biol Chem 266:14964–14969, 1991.
137. Baccarini M, Sabatini DM, App H, Rapp UR, Stanley ER: Colony stimulating factor-1 (CSF-1) stimulates temperature dependent phosphorylation and activation of the RAF-1 proto-oncogene product. EMBO J 9:3649–3657, 1990.
138. Lee RM, Rapp UR, Blackshear PJ: Evidence for one or more Raf-1 kinase kinase(s) activated by insulin and polypeptide growth factors. J Biol Chem 266:10351–10357, 1991.
139. Qureshi SA, Rim M, Bruder J, Kolch W, Rapp U, Sukhatme VP, Foster DA: An inhibitory mutant of c-Raf-1 blocks v-Src-induced activation of the Egr-1 promoter. J Biol Chem 266:20594–20597, 1991.
140. Kolch W, Heidecker G, Lloyd P, Rapp UR: Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. Nature 349:426–428, 1991.

15. Fos and Jun: Inducible transcription factors regulating growth of normal and transformed cells

Jeffrey Holt

Introduction

Although the study of oncogenes has provided some useful insights into cancer mechanisms, the most important benefit from oncogene research has been the delineation of the growth factor response pathway and molecular characterization of important cellular processes. The nuclear proto-oncogenes c-*fos* and c-*jun* have been particularly useful in this regard. Their study has provided important information about gene regulation in response to growth factors, regulation of immediate early genes, and the function and interaction of transcription factors. This chapter will describe (1) the expression and function of these cellular proto-oncogenes as 'immediate early' genes, (2) interactions between Fos, Jun, and other transcription factors, (3) distinct transcriptional effects of Fos, and (4) how mutation and/or overexpression of these oncogenes alters their transcriptional effects and carcinogenic potential, and (5) the potential role of these proteins in human tumorigenesis.

The Fos oncogene was discovered as the cellular homologue of three distinct tumor viruses derived from mice and chickens [1–4]. Both the normal and viral Fos transforming proteins complex with a 39-kD protein [5,6], which was ultimately shown to be the cellular homologue (c-*jun*) of an avian tumor virus oncogene v-*jun* [7]. The fact that these proto-oncogenes form a stable complex is of great importance; it allows these transcription factors to heterodimerize and activate transcription of specific genes. We now know that these genes represent prototypes for a larger gene family that consists of multiple genes that have different expression patterns and transcriptional properties [8]. This gene family is called the b-ZIP or basic zipper family of transcription factors and includes Fos proteins (c-*fos*, Fosb, Fra-1, Fra-2) [9–13], Jun proteins (c-*jun*, JunB, JunD) [14], and a number of CREB/ATF proteins [15–17]. These proteins can heterodimerize with each other in specific combinations, resulting in transcription factors that activate two types of TPA responsive elements: TGAC/GTCA and TGACGTCA [14,17–19]. The members of the Fos subfamily are unique because they can only form heterodimers with Jun or ATF proteins because

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their structure prevents functional homodimers [20,21]. The physical structure of the ancestral basic zipper protein GCN4 has been defined by nuclear magnetic resonance [reviewed in 21].

Fos and Jun are immediate early genes

Growth factors induce quiescent fibroblasts to proliferate by a series of receptor-mediated events, which ultimately require new transcription and protein synthesis [22–24]. However, many of the earliest transcriptional responses to growth factors will occur even in the presence of protein synthesis inhibitors [23,24]. A subset of these growth-factor-inducible genes have been termed *immediate early* genes by virtue of similarities in gene regulation with certain DNA tumor virus genes, notably, rapid but transient transcriptional induction [22,24–26]. The genes c-*fos* and c-*myc* were the first to be identified as immediate early genes after detailed analysis of their mRNA expression patterns. The transient induction of c-*fos* expression is mediated by multiple transacting factors, which bind to the c-*fos* promoter

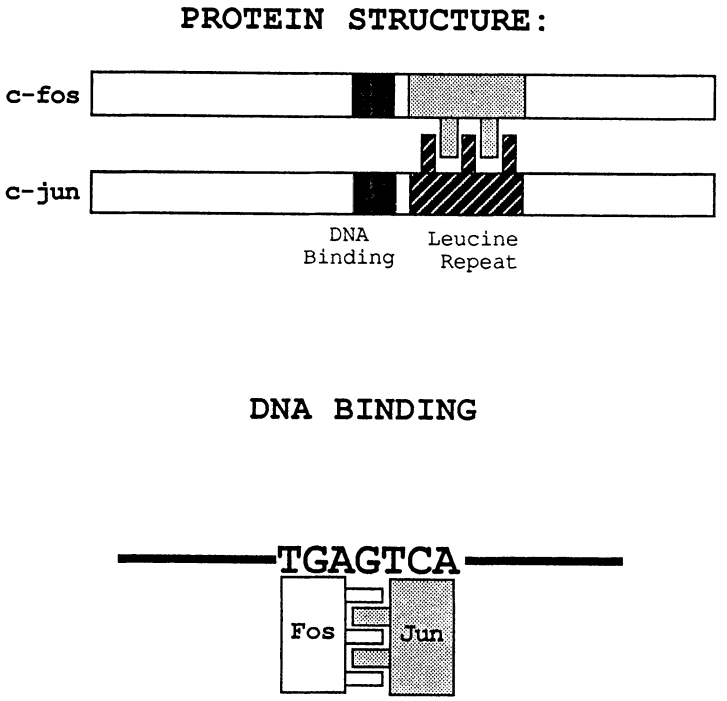

Figure 1. DNA binding by c-*fos* and c-*jun* proteins. The upper panel shows the protein domains of the Fos and Jun proteins, including the DNA binding region and leucine repeat region involved in dimerization of the proteins. The lower panel shows how a Fos-Jun heterodimer presumably binds to its DNA recognition sequence.

and mediate transcriptional activation and repression by mechanisms that are not completely understood [12,27,28]. Immediate early genes have been identified by two primary methods: (1) characterization of proto-oncogene expression patterns (e.g., c-*fos*) and (2) deliberate cloning of these genes by differential cDNA cloning stratagies [24,25]. The c-*jun* gene is also rapidly and transiently induced by growth factors through a combination of transcriptional and posttranscriptional mechanisms [reviewed in 3,20].

Although much of the work on immediate early gene induction has employed cultured fibroblasts as a model system, members of the Fos and Jun gene families are rapidly induced in a number of cells and tissues. The c-*fos* mRNA and its 55-kD nuclear phosphoprotein (Fos) are rapidly but transiently induced by both growth factors and differentiating agents [22, 29–31]. Studies by our laboratory and others have demonstrated that c-*fos* expression is required for fibroblast proliferation and for growth factor-mediated induction of DNA synthesis [32–39]. A complex association between differentiation and c-*fos* expression has been inferred in studies with PC12 pheochromocytoma cells and HL60 leukemia cells [3]. Expression of Fos- and Jun-related proteins is differentially regulated by a variety of agents and stimuli, providing further complexity to the study of immediate early gene action. Finally, Fos proteins are induced during a variety of neural events, including seizures, sensory stimulation, cocaine administration, and circadian responses to light [31]. Induction of Fos and Jun proteins in these diverse biological systems confounds attempts to reconcile the role of these immediate early gene products to a single physiologic function or transcriptional program.

Fos-Jun complexes and AP-1 activity

Studies of the role of Fos and Jun proteins during cell growth and differentiation were greatly facilitated by the discovery that Jun was a component of purified AP-1 preparations [6,11,40–43]. AP-1 represented a previously identified transcription factor that mediates transcriptional induction by tumor promoters [44,45] and has a similar DNA recognition sequence as the homologous yeast transcription factor GCN4 [46,47]. This AP-1 recognition site is present in the adipocyte protein 2 (aP2) promoter, which contains c-*fos* protein in its transcriptional complex [43,48]. Comparison of the protein sequences of c-*fos* and c-*jun* proteins with GCN4 revealed significant sequence homology [8]. The sequence homology of GCN4, AP-1, and aP2 DNA binding sites suggested that Fos and Jun proteins might bind to these DNA sequences. The Fos-Jun heterodimer can bind TRE (TPA-reponsive element: consensus sequence TGAc/gTCA, also called AP-1 recognition site) DNA sequences and activate transcription of target genes that contain these DNA sequences [35,36,39,42,49–51]. Comparison of the protein sequences of Fos and Jun family proteins with

the yeast transcriptional activator GCN4 revealed a repeated leucine heptad motif, which was initially termed the *leucine zipper*, but is now presumed to represent a coiled-coil interaction [52]. This leucine motif is required for heterodimerization of Fos and Jun proteins, and consequent DNA binding. In vitro synthesis of Fos and Jun proteins in reticulocyte lysates demonstrated that Fos could only bind DNA (the AP-1 binding site or TRE: consensus TGAGTCA) following heterodimerization with Jun [14,19,42,53–58]. Jun can bind DNA as a homodimer, but heterodimarization with Fos may increase the affinity of this binding for the AP-1 DNA binding site. Mutations in the leucine motif of Fos disrupt dimerization and DNA binding, while mutations in the basic alpha-helical region disrupt DNA binding but permit Jun association [50,55,59–62]. The structure of these functional domains within the Fos and Jun proteins is illustrated in Fig. 1.

The model that the alpha-helical domain controls DNA binding and the leucine repeat controls Fos-Jun heterodimerization is extremely important, but these is evidence that other factors are involved: (1) a sulfhydryl reduction activity apparently modifies cysteine residues in Fos and/or Jun proteins prior to DNA binding [20,63]; (2) an inhibitory factor may bind to a region of Jun protein [64,65]; (3) accessory molecules have been described that inhibit AP-1 activity [66], further complicating the regulatory possibilities. Fos protein has been demonstrated to participate in complex interactions with other transcription factors. Several laboratories have demonstrated that Fos and Jun proteins may interact with steroid hormone receptors to increase the flexibility of transcriptional responses [67–69]. These studies taken together provide support for the idea that Fos protein may function as a modulator of other transcriptional programs by interacting with a variety of transcription factors.

Transrepression by Fos protein

The rapid but transient induction of the c-*fos* gene is mediated by multiple transacting factors that bind to the c-*fos* promoter and mediate transcriptional activation and repression by complex mechanisms that are not yet completely understood. Inhibitors of protein synthesis, such as cycloheximide, produce a sustained induction of immediate early genes (including c-*fos*), in marked contrast to the usual transient induction observed in untreated cells [23,24]. Although numerous models could be proposed to explain the transient nature of immediate early gene induction and its prolongation and enhancement by cycloheximide, a popular notion suggests that one or more of the immediate early gene products prevents sustained transcription of itself and/or similar genes [12]. Two distinct lines of evidence indicate that Fos protein autoregulates its own production:

1. Overexpression of Fos inhibits transcription mediated by the SRE (serum response element) within the c-*fos* and Egr-1 promoters [12,70–73]

2. Underexpression of Fos employing antisense RNA methods activates and prolongs transcription of the c-*fos* promoter at the SRE and adjacent sites [74].

The c-*fos* promoter element(s) responsible for autoregulation is in some dispute, with different studies proposing either classical Fos-Jun binding sites, AP-1/ATF [12,75], or the serum response element, SRE [71,72,74, 76,77]. The serum response element was identified by mutational analysis of the c-*fos* promoter and is sufficient to confer serum induction upon a heterologous gene [78,79]. The serum response element represents the binding site for serum response factors, nuclear protein(s) whose binding is essential for growth factor induction of the c-*fos* gene [27,58,72,76,80]. In addition to the serum response factor, other nuclear proteins have been identified that interact with adjacent upstream promoter elements [12,81]. The in vivo significance of these adjacent elements is unclear because the 14-bp inner core of the SRE is sufficient for both induction and repression of c-*fos* transcription [77].

Which functional domains of Fos are necessary for transrepression? Although the helical DNA binding domain is dispensable, studies differ on whether the leucine zipper domain is required. Fos mutants with large deletions or major changes in the leucine repeat domain cannot repress [72]. However, a small mutation within the leucine heptad repeat of Fos eliminates Fos-Jun association with little effect on SRE transrepression [70]. This suggests that SRE transrepression by Fos does not require heterodimerization with Jun or another protein capable of binding DNA. The role of the C-terminus in transrepression is also in some dispute. Deletion of C-terminal amino acid sequences have little effect on transrepression; only insertions within this region can alter the protein's ability to transrepress [47,71]. However, studies involving site-directed mutation of the c-*fos* protein indicate that phosphorylation of the Fos C-terminus is apparently required for transrepression, although the introduction of net negative charges may offset this requirement [73].

Differential transcriptional regulation by Fos and Jun family members

Fos-Jun transcriptional response is futher complicated by the presence of specific transcriptional inhibitors, including members of the Fos and Jun families. The JunB protein can only bind multimerized AP-1 sites and apparently functions as a negative regulator of Fos/Jun transcriptional activity [82,83]. The FosB gene codes for two distinct proteins, one of which is C-terminally truncated (ΔFosB) and functions as an inhibitor of Fos/Jun transcriptional activity [13,19], although this result may be considered controversial [10]. Other inhibitors of AP-1 activity have also been identified, although the corresponding genes have not yet been cloned [65,66].

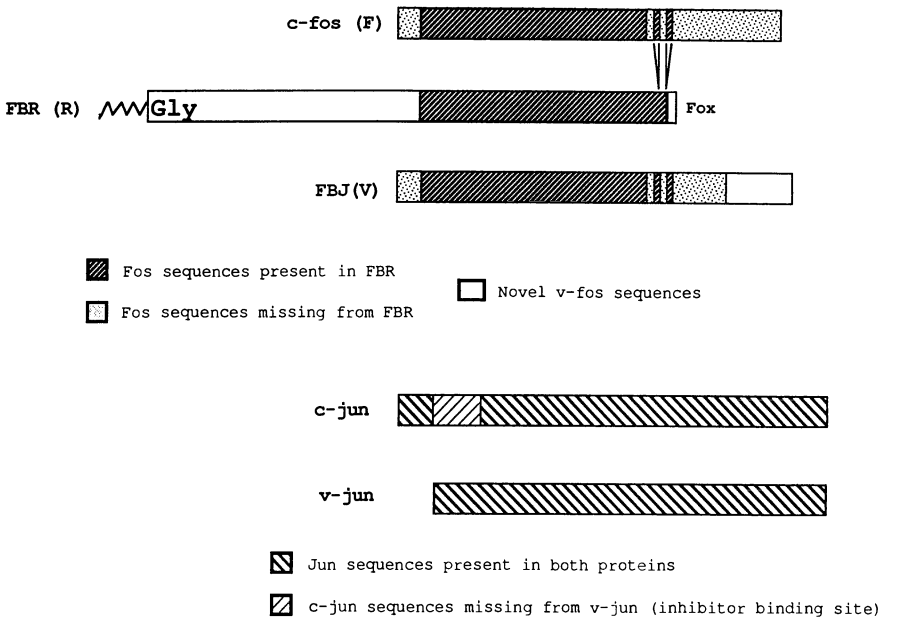

Figure 2. Structural differences between c-*onc* and v-*onc* proteins. The upper panel shows the structural differences between the c-*fos* proto-oncogene and two of its transforming viral homologues: FBR and FBJ. The open boxes show regions of unique viral sequences that are not present in c-*fos*: FBR differs at both the N- and C-termini, including its N-terminal myristylation (denoted by the zigzag line); FBJ differs due to a C-terminal frameshift. Differences between c-*jun* and v-*jun* are shown in the lower panel.

Mechanisms of tumorigensis by v-*fos* and v-*jun*

Both c-*fos* and c-*jun* genes can transform at low efficiencies but their mutant counterparts generally have increased transformation potential [84,85]. Because v-*fos* and v-*jun* are much more effective transforming genes than their corresponding proto-oncogenes, genetic methods have been employed to determine the functional domains and molecular mechanisms responsible for transformation by v-*fos* and v-*jun*. The structures of the three v-*fos* and the only v-*jun* transforming protein are illustrated in Fig. 2. FBR protein differs from the others because it can both transform and immortalize primary cells. The FBJ v-*fos* protein binds AP-1 DNA sites and activates transcription at the TRE but cannot transrepress the SRE within the c-*fos* promoter [54,71]. The molecular functions of the FBR v-*fos* protein are more complex. FBR v-*fos* protein is myristylated as a consequence of the fusion of the viral *gag* sequence to mouse *fos* sequences. This myristylation has previously been shown to inhibit TRE transactivation by FBR (*gag-fos*) protein [54] and SRE transrepression by FBR protein [71]. Transfer of myristylation sequences to c-*fos* is sufficient to inhibit TRE transactivation

but is not sufficient to inhibit transrepression by Fos, suggesting a functional difference between the FBR and Fos C termini. Analysis of Fos mutants and Fos/FBR chimeras indicated that the FBR C terminus encodes a novel transrepressor domain, which is functionally competent but inhibited by N-myristylation [71]. These results indicate that both N-terminal and C-terminal mutations contribute to the transrepressional properties of FBR v-*fos* protein. Although clear differences in transcriptional regulation are apparent when v-*fos* proteins are compared with c-*fos* protein [54,71,86], it is not yet clear whether these differences contribute to transformation by v-*fos*.

V-*jun* differs structurally and functionally from c-*jun* primarily because v-*jun* lacks 27 amino acids, which represent the proposed binding site for a tissue-specific inhibitor of AP-1 activity (Fig. 2). The loss of these regulatory sequences may lead to uncontrolled transcriptional activity by v-*jun* [65]. Although these differences in transcriptional properties between c-*onc* and v-*onc* are intriguing, there is still no direct evidence that v-*jun* or v-*fos* proteins transform as a consequence of alterations in cellular AP-1 transcription.

Possible role of Fos and Jun in human tumors

Although mutations in Fos or Jun genes have not been identified in human cancer cells, there is accumulating evidence that these transcription factors may serve as nuclear targets for the effects of cytoplasmic oncogenes, such as *ras* mutations. The concept of cooperating oncogenes developed from the observation that cytoplasmic 'transforming' oncogenes, such as v-*src* or v-*ras*, could not transform primary cells in the absence of 'immortalizing' nuclear oncogenes. Recent studies indicate that expression of these 'transforming' oncogenes produces a site-specific dephosphorylation of Jun protein, resulting in an increased Jun-mediated transactivation [29, 87]. Transforming oncogenes also produce an increased phosphorylation by MAP kinase of distinct residues, providing another potential mechanism for increased AP-1 activity [88]. A third mechanism for this oncogene cooperation is provided by a report that transforming oncogenes alleviate repression by an inhibitor of AP-1 activity [64,65]. These studies indicate that transformation by cytoplasmic oncogenes may result in increased AP-1 activity and may presumably alter the expression of growth-regulatory genes.

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References

1. Nishizawa M, Goto N, Kawai S: An avian transforming retrovirus isolated from a nephroblastoma that carries the fos gene as the oncogene. J Virol 61:3733–3740, 1987.
2. Van Beveren C, van Straaten F, Curran T, Muller R, Verma IM: Analysis of FBJ-MuSV provirus and c-fos (mouse) gene reveals that viral and cellular fos gene products have different carboxy termini. Cell 32:1241–1255, 1983.
3. Verma IM, Sassone-Corsi P: The c-fos protooncogene. Cell 51:513–514, 1988.
4. Ward JM, Young DM: Histogenesis and morphology or periosteal sarcomas induced by FBJ virus in NIH swiss mice.
5. Curran T, VanBeveren C, Ling N, Verma IM: Viral and cellular fos proteins are complexed with a 39,000 dalton cellular protein. Mol Cell Biol 5:167–172, 1985.
6. Sassone-Corsi P, Lamph WW, Kamps M, Verma IM: Fos associated cellular p39 is related to nuclear transcription factor AP-1. Cell 54:553–560, 1988.
7. Maki Y, Bos TJ, Davis C, Starbuck M, Vogt PK: Avian sarcoma virus 17 carries the jun oncogene. Proc Natl Acad Sci USA 84:2848–2852, 1987.
8. Landshulz, Johnson PF, McKnight SL: The leucine zipper: A hypothetical structure common to a new class of DNA binding protein. Nature 240:1759–1764, 1988.
9. Cohen DR, Curran T: Fra-1: A serum-inducible cellular immediate early gene that encodes a fos-related antigen. Mol Cell Biol 8:2063–2069, 1988.
10. Dobrzanski P, Noguchi T, Kovary K, Rizzo CA, Lazo PS, Bravo R: Both products of the fosB gene, FosB and its short form, FosB/SF, are transcriptional activators in fibroblasts. Mol Cell Biol 11:5470–5478, 1991.
11. Franza RB, Jr, Rauscher III FJ, Josephs SF, Curran T: The fos complex and fos-related antigens recognize sequence elements that contain AP-1 binding sites. Science 239:1150–1153, 1988.
12. Sassone-Corsi P, Sisson JC, Verma IM: Transcriptional autoregulation of the protooncogene fos. Nature 334:314–319, 1988.
13. Yen J, Wisdom RM, Tratner I, Verma IM: An alternatively spliced form of FosB is a negative regulator of transcriptional activation and transformation by Fos proteins. Proc Natl Acad Sci USA 88:5077–5081, 1991.
14. Nakabeppu Y, Ryder K, Nathans D: DNA binding activities of three murine jun proteins: Stimulation by fos. Cell 55:907–915, 1988.
15. Benbrook DM, Jones NC: Heterodimer formation between CREB and Jun proteins. Oncogene 5:295–302, 1990.
16. Hai T, Curran T: Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. Proc Natl Acad Sci USA 88:3720–3724, 1991.
17. Macgregor PF, Abate C, Curran T: Direct cloning of leucine zipper proteins: Jun binds cooperatively to the CRE with CRE-BP1. Oncogene 5:451–458, 1990.
18. Ivashkiv JB, Liou H-C, Kara CJ, Lamph WW, Verma IM, Glimcher LH: mXBP/CRE-Bp2 and c-Jun form a complex which binds to the cyclin AMP, but not to the 12-O-tetradecanoylphorbol-13-acetate, response element. Mol Cell Biol 10:1609–1621, 1990.
19. Nakabeppu Y, Nathans D: A naturally occurring truncated form of FosB that inhibits Fos/Jun transcriptional activity. Cell 64:751–759, 1991.
21. Harrison SC: A structural taxonomy of DNA-binding domains. Nature 353:715–719, 1991.
22. Greenberg ME, Ziff EB: Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature 311:433–438, 1984.
23. Greenberg ME, Hermanowski AL, Ziff EB: Effect of protein synthesis inhibitors on growth factor activation of c-fos, c-myc, and actin gene transcription. Mol Cell Biol 6:1050–1057, 1986.
24. Lau LF, Nathans D: Expression of a set of growth related immediate early genes in Balbc/3T3 cells: Coordinate regulation with c-fos or c-myc. Proc Natl Acad Sci USA 80:4271–4275, 1987.

25. Almendral JM, Sommer D, MacDonald-Bravo H, Burckhardt J, Perera J, Bravo, R: Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol Cell Biol 8:2140–2148, 1988.
26. Chavirer P, Janssen-Timmen U, Mattei M-G, Zerial M, Bravo R, Charnay P: Structure, chromosome location, and expression of the mouse sinc-finger gene Krox-20: Multiple gene products and coregulation with the proto-oncogene c-fos. Mol Cell Biol 9:787–797, 1989.
27. Herrera RE, Shaw PE, Nordheim A: Occupation of the c-fos serum response element in vivo by a multi-protein complex is unaltered by growth factor induction. Nature 340:68–70, 1989.
28. Norman C, Runswick M, Pollock R, Treisman R: Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. Cell 55:989–1003, 1988.
29. Binetruy B, Smeal T, Karin M: Ha-ras augments c-jun activity and stimulates phosphorylation of its activation domain. Nature 351:122–127, 1991.
30. Jackson JA, Holt JT, Pledger WJ: Continuous exposure to PDGF is required for growth induction: Correlation with post-translational stability of Fos. J Biol Chem 267:17444–17448, 1992.
31. Rusack B, Robertson H, Wisden W, Hunt S: Light pulses that shift rhythms induce gene expression in the suprachiasmatic nucleus. Science 248:1237–1239, 1990.
32. Holt JT, Gopal TV, Moulton AD, Nienhuis AW: Inducible production of c-fos anti-sense RNA inhibits 3T3 cell proliferation. Proc Natl Acad Sci USA 83:4794–4798, 1986.
33. Holt JT, Nienhuis AW: c-fos proto-oncogene expression is necessary for normal growth of mouse 3T3 cells. UCLA Symp Mol Cell Biol 51:313–320, 1988.
34. Holt JT: Growth inhibition by antisense nucleic acids. In: Brakel C (ed): Discoveries in Antisense Nucleic Acids, Vol. 20, Gulf Press, 1989, pp 81–94.
35. Kerr LD, Holt JT, Matrisian LM: Growth factors regulate transin gene expression by c-fos dependent and c-fos independent pathways. Science 242:1424–1427, 1988.
36. Kim S-J, Denhez F, Kim K-Y, Holt JT, Sporn MB, Roberts AB: Activation of the second promoter of the TGF-beta1 gene by TGF-beta1 and phorbol ester occurs through the same target sequences. J Biol Chem 264:19373–19378, 1989.
37. Nishikura K, Murray JM: Antisense RNA of proto-oncogene c-fos blocks renewed growth of quiescent 3T3 cells. Mol Cell Biol 7:639–649, 1987.
38. Riabowol KT, Vosatka RJ, Ziff EB, Lamb NJ, Feramisco JR: Microinjection of fos-specific antibodies blocks DNA synthesis in fibroblast cells. Mol Cell Biol 8:1670–1676, 1988.
39. Schonthal A, Herrlich P, Rahmsdorf, Ponta H: Requirement for fos gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. Cell 54:325–334, 1988.
40. Angel P, Allegretto EA, Okino S, Hattori K, Boyle WK, Hunter T, Karin M: The jun oncogene encodes a sequence-specific transactivator similar to AP-1. Nature 332:166–171, 1988.
41. Bohmann D, Bos TJ, Admon A, Nishimura T, Vogt PK, Tjian R: Human protoncogene c–jun encodes a DNA-binding protein with structural and functional properties of transcription factor AP-1. Science 238:1386–1392, 1987.
42. Chiu R, Boyle WJ, Meek J, Smeal T, Hunter T, Karin M: The c-fos protein interacts with c-jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell 54:541–552, 1988.
43. Rauscher FJ, III, Sambucetti LC, Curran T, Distel RJ, Speigelman BM: Common DNA binding site for fos protein complexes and transcription factor AP-1. Cell 52:471–480, 1988.
44. Angel P, Imagawa M, Chiu R, Stein B, Imbra TJ, Rahmsdorf HJ, Jonat C, Herrlich P, Karin M: Phorbol ester inducible genes contain a common cis element recognized by a TPA-modulated transacting factor. Cell 49:729–739, 1987.
45. Lee W, Mitchell P, Tjian R: Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 49:741–752, 1987.

46. Hope IA, Struhl K: GCN4, a eukaryotic transcriptional activator protein, binds as a dimer to target DNA. EMBO J 6:2781–2784, 1987.
47. Wilson T, Treisman R: Fos C-terminal mutations block downregulation of c-fos transcription following serum stimulation. EMBO J 7:4193–4202, 1988.
48. Distel RJ, Ro HS, Rosen BS, Groves DL, Spiegelman BM: Nucleoprotein complexes that regulate gene expression in adipocyte differentiation: Direct participation of c-fos. Cell 49:835–844, 1987.
49. Kerr LD, Miller DB, Matrisian LM: TGF-beta inhibition of transin/stromyelysin gene expression is mediated through a Fos binding sequence. Cell 61:267–278, 1990.
50. Sassone-Corsi P, Ransone LJ, Lamph WW, Verma IM: Direct interactions between fos and jun nuclear oncopproteins: Role of the leucine zipper domain. Nature 336:690–692, 1988.
51. Sonnenberg JL, Rauscher III FJ, Morgan JI, Curran T: Regulation of proenkephalin by Fos and Jun. Science 246:1622–1625.
52. O'Shea EK, Rutkowski R, Kim PS: Evidence that the leucine zipper is a coiled coil. Science 243:538–542, 1989.
53. Halazonetis TD, Goergopoulos K, Greenberg ME, Leder P: C-jun dimerizes with itself and with c-fos forming complexes of different DNA binding affinities. Cell 55:917–924, 1988.
54. Kamata N, Jotte R, Holt JT: Myristylation alters DNA binding and transactivation of FBR (gag-fos) protein. Mol Cell Biol 11:765–772, 1991.
55. Kouzarides T, Ziff E: The role of the leucine zipper in the fos-jun interaction. Nature 336:646–651, 1988.
56. Melton DA, Krieg DA, Rebagliati MR, Flaniatis T, Zinn K, Green MR: SP6 RNA polymerase. Nucleic Acid Res 12:7035–7056, 1984.
57. Neuberg M, Schuermann M, Humter JB, Muller R: Two functionally different regions in fos are required for the sequence-specific DNA interaction of the fos/jun complex. Nature 338:589–590, 1989.
58. Rauscher FJ, III, Voulalas PJ, Franza BR, Curran T: Fos and jun bind cooperatively to the AP-1 site: Reconstitution in vitro. Genes Dev 2:1687–1699, 1988.
59. Gentz R, Rauscher FJ, III, Abate C, Curran T: Parallel Association of fos and jun leucine zippers juxtaposes DNA binding domains. Science 243:1695–1699, 1989.
60. Schuerman M, Neuberg M, Hunter JB, Jenuwein T, Ryseck RP, Bravo R, Muller R: The leucine repeat motif in fos protein mediates complex formation with jun/AP-1 and is required for transformation. Cell 56:507–516, 1989.
61. Struhl K: The DNA binding domain of the jun oncoprotein and the yeast GCN4 transcriptional activator are functionally homologous. Cell 50:841–846, 1987.
62. Turner R, Tjian R: Leucine repeats and an adjacent DNA binding domain mediate the formation of functional c-fos c-jun heterodimers. Science 243:1689–1694, 1989.
63. Abate C, Patel L, Rauscher FJ, III, Curran T: Redox regulation of Fos and Jun DNA binding activity in vitro. Science 249:1157–1161, 1990.
64. Baichwal VR, Park A, Tjian R: v-src and EK ras alleviate repression of c-jun by a cell-specific inhibitor. Nature 352:165–168, 1991.
65. Bohman D, Tjian R: Biochemical analysis of transcriptional activation by Jun: Differential activity of c- and v-Jun. Cell 59:709–717, 1989.
66. Auwerx J, Sassone-Corsi P: IP-1: A dominant inhibitor of Fos/Jun whose activity is modulated by phosphorylation. Cell 64:983–993, 1991.
67. Diamond MI, Miner JN, Yoshinaga SK, Yamamoto KR: Transcription factor interactions: Selectors of positive or negative regulation from a single DNA element. Science 249:1266–1272, 1990.
68. Jonat G, Rahmsdorf HJ, Park KK, Cato ACB, Gebel S, Ponta H, Herrlich P: Antitumor promotion and antinflammation: Downmodulation of AP-1 (Fos-Jun) activity by glucocorticoid hormone. Cell 62:1189–1204, 1990.
69. Yang-Yen HF, Chambard JC, Sun YL, Smeal T, Schmidt TJ, Drouin J, Karin M:

Transcriptional interference between c-Jun and the glucocorticoid receptor: Mutual inhibition of DNA binding due to direct protein-protein interaction. Cell 62:1205–1215, 1990.
70. Gius D, Cao X, Rauscher FJ, III, Cohen DR, Curran T, Sukhatme VP: Transcriptional activation and repression by Fos are independent functions: The C terminus represses immediate early gene expression via CarG elements. Mol Cell Biol 10:4243–4255, 1990.
71. Kamata N, Holt JT: Inhibitory effect of myristylation on transrepression by FBR (gag-fos) protein. Mol Cell Biol 12:876–882, 1992.
72. Lucibello FC, Lowag C, Neuberg M, Muller R: Transrepression of the mouse c-fos promoter: A novel mechanism of fos-mediated trans-regulation. Cell 49:741–752, 1989.
73. Ofir R, Dwarki VJ, Rashid D, Verma IM: Phosphorylation of the C-terminus of Fos protein is required for transcriptional transrepression of the c-fos promoter. Nature 348:80–82, 1990.
74. Robinson-Benion C, Kamata N, Holt JT: Antisense mapping of the c-fos promoter: Role of the serum response element. Antisense Res Dev 1:21–33, 1991.
75. Schonthal A, Buscher M, Angel P, Rahmsdorf HJ, Ponta H, Hattori K, Chiu R, Karin M, Herrlich P: The fos and Jun/AP-1 proteins are involved in the downregulation of Fos transcription. Oncogene 4:629–636, 1989.
76. Konig H, Ponta H, Rahmsdorf U, Buscher M, Schonthal A, Rahmsdorf HJ, Herrlich P: Autoregulation of fos: The dyad symmetry element as the major target of repression. EMBO J 8:25592–2566, 1989.
77. Rivera VM, Sheng M, Greenberg ME: The inner core of the serum response element mediates both the rapid induction and subsequent repression of c-fos transcription following serum stimulation. Genes Dev 4:255–268, 1990.
78. Deschamps J, Meijlink F, Verma IM: Identification of a transcriptional enhancer element upstream from the proto-oncogene fos. Science 230:1174–1177, 1985.
79. Treisman R: Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5′ element and c-fos 3′ sequences. Cell 42:889–902, 1985.
80. Shaw PE, Frisch S, Nordheim A: Repression of c-fos transcription is mediated through p67SRF bound to the SRE. EMBO J 8:2567–2574, 1989.
81. Gilman MZ, Wilson RN, Weinberg RA: Multiple protein binding sites in the 5′-flanking region regulate c-fos expression. Mol Cell Biol 6:4305–4315, 1986.
82. Chiu R, Angel P, Karin M: JunB differs in its biological properties from and is a negative regulator of c-jun. Cell 59:979–986, 1989.
83. Schutte J, Viallet J, Nau M, Segal S, Fedorko J, Minna J: JunB inhibits and c-fos stimulates the transforming and transactivating activities of c-jun.
84. Meijlink F, Curran T, Miller AD, Verma IM: Removal of a 67 base pair sequence in the noncoding region of proto-oncogene fos converts it to a transforming gene. Proc Natl Acad Sci USA 82:4987–4991, 1985.
85. Schutte J, Minna JD, Birrer MJ: Deregulated expression of human c-jun transforms primary rat embryo cells in cooperation with an activated c-Ha-ras gene and transforms Rat-1a cells as a single gene. Proc Natl Acad Sci USA 86:2257–2261, 1989.
86. Jotte RM, Kamata N, Holt JT: Manuscript in preparation.
87. Boyle WJ, Smeal T, Defize LHK, Angel P, Woodgett JR, Karin M, Hunter T: Activation of protein kinase C decreases phosphorylation of c-jun at sites that negatively regulate its DNA binding activity. Cell 64:573–584, 1991.
88. Pulverer BJ, Kryiakis JM, Avruch J, Nikolakaki E, Woodgett JR: Phosphorylation of c-jun mediated by MAP kinases. Nature 353:670–674, 1991.

16. DNA binding by the Myc Oncoproteins

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Introduction

The v-*myc* oncogene was first identified in 1977 as the transforming gene of the MC29 avian retrovirus, which causes myelocytomatosis, carcinomas, and sarcomas in chickens [1]. The c-*myc* proto-oncogene has since been identified in a wide variety of organisms ranging from the invertebrate sea star [2] to humans [3]. Deregulated expression of a normal c-*myc* gene leads to malignant transformation in certain cell culture models, such as primary rat embryo fibroblasts (REF; in which the coexpression of a second activated oncogene, *ras*, is also required) [4,5], and in transgenic mice [6] and rabbits [7]. Burkitt lymphoma, a naturally occurring human tumor, provides a paradigm for the role of c-*myc* in malignant transformation. In this case, chromosomal translocation of the c-*myc* locus to a location downstream of the regulatory elements of the immunoglobulin heavy chain gene results in deregulated expression of c-*myc* [8]. In contrast to many other oncogenes that have activating mutations in the coding sequence, this deregulated expression of a normal c-*myc* coding sequence appears to be responsible for the oncogenic contribution of c-*myc* [4].

The product of the c-*myc* oncogene, with translation initiated at a conventional ATG start codon, is a 439 amino acid nuclear phosphoprotein with a short half-life of 15–30 minutes [reviewed in 9]. Translation from an upstream alternative CTG start codon gives rise to a 453 amino acid protein [10]. It is widely expressed in virtually all proliferating tissues. In resting cells in culture, c-Myc is expressed at low levels, but mitogenic stimulation induces high levels of c-Myc expression within minutes. These properties have suggested that c-Myc may serve as a master nuclear regulatory protein, controlling subsequent cellular events necessary for proliferation. The hypothesis that c-Myc is involved in the regulation of growth-related genes has led to the proposal that c-Myc might be a transcription regulatory factor [reviewed in 11,12].

Unlike many other transcription factors, c-Myc contains two different DNA binding motifs. The first, the basic/helix-loop-helix/leucine zipper (bHLH-ZIP) motif [13], confers sequence-specific DNA recognition [14,15]

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and is critical for *c-nyc* function [16]. Highly homologous bHLH-ZIP motifs are found in N-Myc, the product of the N-*myc* oncogene that is amplified in human neuroblastoma cells [17,18]; L-Myc, the product of the L-*myc* oncogene that is amplified in human small cell lung carcinoma cells [19]; v-Myc, the product of the avian retroviral homolog of chicken c-*myc*; and Max, a protein that can heterodimerize with Myc family members [20]. The second DNA binding motif, the 'SPXX-like' motif [21], provides nonspecific DNA binding (NDB) function [22] and is not absolutely required for c-Myc cotransforming activity [16]. This chapter will reinforce the concept that the c-Myc protein is a potential transcription factor, with a detailed discussion of DNA binding function by the Myc family of proteins.

Functional domains of c-Myc

A number of specific properties have been shown to be associated with various domains of the c-Myc protein (Fig. 1) [reviewed in 23]. Initial studies mapped the regions of c-Myc that are necessary for its ability to transform REF cells in cooperation with an activated *ras* gene [16,24]. These experiments demonstrated that the amino-terminal 143 amino acids and the carboxyl-terminal 89 amino acids are necessary for transformation. Subsequent investigations have revealed a structure common to many transcription factors: a transcriptional activation domain (TAD) capable of stimulating transcription of a target gene and a specific DNA-binding domain, which confers specificity to transcription by mediating binding of the protein to a specific DNA sequence, usually upstream of the target gene. The TAD of c-Myc overlaps precisely with the amino terminal 143 amino

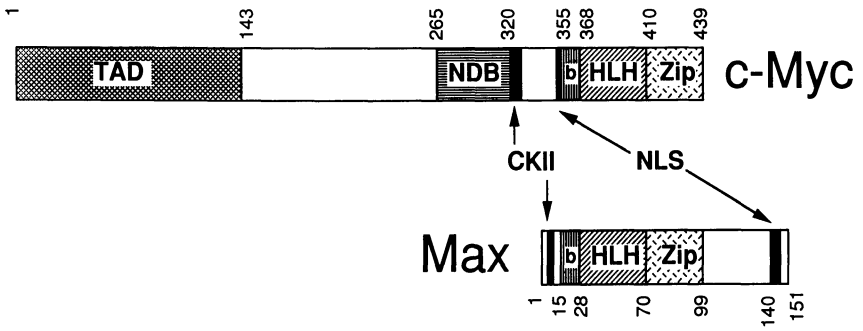

Figure 1. Functional domains of c-Myc and Max. Each bar schematically depicts the localization of various identified functional domains of the c-Myc and Max proteins. Numbers indicate amino acid residues beginning at the amino terminus. TAD = transcriptional activation domain; NDB = nonspecific DNA binding domain; b = basic region; HLH = helix-loop-helix; Zip = leucine zipper; CK II = sites of phosphorylation by casein kinase II; NLS = nuclear localization signal.

acid transformation domain [25]. Similarly, the bHLH-ZIP motif and sequence-specific DNA binding function coincide with the carboxyl-terminal transformation domain (amino acids 350–439) [13–15]. These data suggest that transcriptional activation and DNA binding are integral to the function of c-Myc in malignant transformation.

There are other functional domains in the c-Myc protein. The nonspecific DNA binding domain is located between amino acids 265 and 318 [22]. A nuclear localization signal (amino acids 320–328) efficiently targets the protein into the nucleus [26]. Neither of these domains is strictly required for transformation by c-Myc [16]. The potential significance of different DNA binding functions with respect to transformation will be discussed below.

Sequence-specific DNA binding by Myc and other bHLH proteins

Dimerization via the HLH and ZIP motifs

The bHLH motif is a hypothetical secondary protein structure, consisting of (1) the basic region: an alpha-helix rich in basic amino acids, approximately 12 amino acids in length; (2) helix 1: an alpha-helix of approximately 12 residues with amphipathic characteristics, i.e., one face of the helix is predominantly composed of hydrophobic residues and the opposite face is composed primarily of hydrophilic residues; (3) the loop: a region without definite structural characteristics, 10–12 residues in length; and (4) helix 2: a second amphiphatic alpha-helix region [13]. The HLH motif serves as a dimerization interface between two HLH proteins, most often two different HLH proteins in a heterodimeric combination [27]. Biochemical and physicochemical data suggest that the hydrophobic faces of the two helices of each subunit interact in a parallel four-helix bundle to provide a stable protein–protein interaction [28,29]. Hypothetically, the paired basic regions of the two proteins extend from the four-helix bundle in a forklike structure (Fig. 2). The basic regions may then potentially lie in the major groove of DNA, making specific contacts with DNA [30]. Dimerization through the HLH is necessary for DNA sequence recognition and binding by the adjacent basic region in this class of proteins [13].

A subset of the bHLH protein family is the bHLH-ZIP group of proteins, which include the Myc and Max proteins, as well as the known transcriptional activators TFE3 [31], USF [32], and AP4 [33]. These proteins are distinguished by a leucine zipper (ZIP), which directly extends from helix 2 of the bHLH. The leucine zipper is an amphipathic alpha-helical motif, in which leucine residues are present every seven amino acid residues, forming the major component of the hydrophobic face [34]. Although the ZIP serves as the sole dimerization motif in some proteins (Fos, Jun, C/EBP, etc.), in the bHLH-ZIP family the ZIP appears to be an extension of helix 2, likely

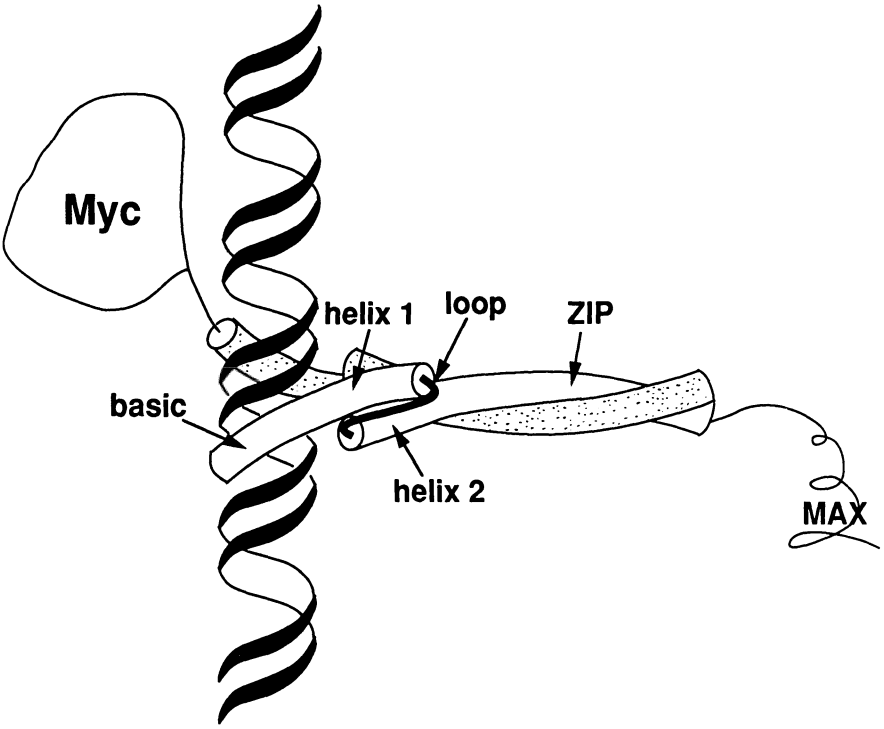

Figure 2. Schematic depiction of c-Myc/Max heterodimer contacting a specific DNA sequence. Shown is a representation of the hypothetical four-helix bundle arrangement of the bHLH-ZIP regions. The c-Myc portion of the dimer is shaded.

providing additional stability and specificity to the dimer formation [35].

The c-Myc protein is unable to bind to DNA as a homodimer in vitro [36]. A polypeptide containing the minimal bHLH-ZIP domain of c-Myc is able to homodimerize and bind DNA, but this in vitro phenomenon using a truncated protein is unlikely to reflect the function of the full-length protein in vivo [37]. Association of c-Myc with Max, however, yields a heterodimer that binds to DNA effectively in vitro, and it is likely that this dimer is biologically active [20,36,38]. Max homodimers also bind to the same DNA sequence, and this may be of biological importance (see below). Likewise, Max is able to dimerize with N-Myc and L-Myc proteins, although none of these proteins appears to interact with USF or AP4 [20,39].

Amino acid–nucleotide interaction via the basic region

The c-Myc/Max heterodimer and the Max homodimer bind to DNA bearing the core palindromic hexanucleotide CACGTG [20,36,38]. These complexes also bind to the sequence CACATG, but with lower affinity. A number of other bHLH proteins also bind to CACGTG; these are designated class B

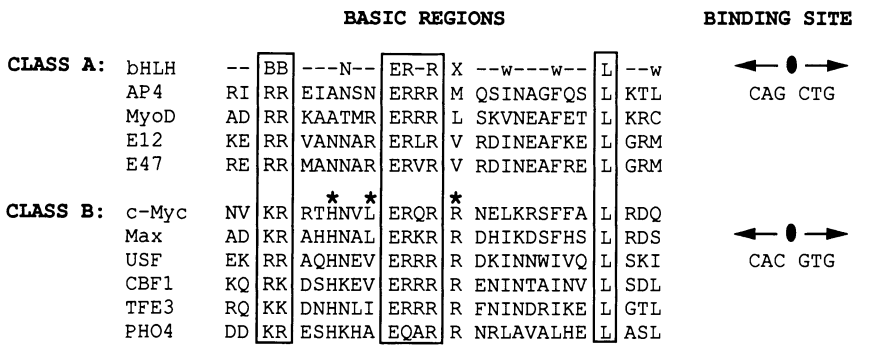

Figure 3. Amino acid homologies and differences in the basic regions of bHLH family members. Partial amino acid sequences of nine bHLH proteins are aligned at their basic regions, displayed by a single-letter amino acid code. Boxes indicate residues conserved in all bHLH proteins. Asterisks mark the positions of the conserved histidine, hydrophobic, and arginine residues in class B bHLH proteins. B = basic; X = any amino acid; w = hydrophobic residue (figure adapted from ref. 40, with permission.).

bHLH proteins. Class A bHLH proteins prefer the sequence CAGCTG or CAGGTG [40]. Thus, all known bHLH proteins bind to DNA sequences with the pattern CANNTG, where N is any nucleotide [41]. A comparison of the basic regions of the bHLH proteins reveals a number of highly conserved amino acids, which are presumably involved in the recognition of the conserved nucleotides, the initial CA and the final TG (Fig. 3).

Several amino acid residues that are conserved among class B (but not class A) proteins are likely to be involved in distinguishing between CACGTG and CAGCTG. These residues — histidine, valine, and arginine — are indicated in Fig. 3. Arginine 367 of c-Myc has, in fact, been shown to be essential in dictating sequence specificity. Mutation of the comparable residue (Met to Arg) in the basic region of AP4, a class A bHLH protein, changes its specificity to that of the class B sequence CACGTG [40]. The conserved histidine and valine in class B proteins probably also serve important functions with regard to specific DNA binding, but these have not been determined.

In addition to the core hexanucleotide, the three flanking residues upstream and downstream are important to DNA binding specificity [42]. A 12-nucleotide nearly palindromic binding site has been determined for purified native c-Myc protein: (G/A)ACCACGTGCTC [43].

Functional consequences of specific DNA binding

In certain cell culture systems, a consequence of DNA binding by c-Myc is transformation of the cells to a malignant phenotype [4,5,44]. The importance of DNA binding in transformation by c-Myc has been inferred from a variety of data. First, mutations in the bHLH-ZIP region of c-Myc

abolish both its ability to bind DNA specifically in vitro and to transform cultured cells [16,36,45]. Second, certain mutations in c-Myc result in proteins that not only are inactive for transformation, but also are able to inhibit transformation by wild-type c-Myc in a *trans*-dominant fashion, i.e., the presence of a mutant gene can neutralize the transforming effect of a wild-type gene [45]. A mutant c-Myc protein that lacks part of the c-Myc TAD retains the ability to dimerize, presumably with Max or Max-like proteins, and to bind to DNA. This dimer is able to compete with wild-type dimers for DNA binding but fails to stimulate transcription from those DNA sites. Hence concurrent expression of the mutant with wild-type c-Myc fails to result in transformation of REF cells. A second type of *trans*-dominant mutant c-Myc contains a small insertion mutation near the basic region (45a, C.V. Dang, unpublished). Such a mutation permits its dimerization with Max, but the heterodimer is unable to bind to DNA because of the defect in the DNA contact region. This mutant also antagonizes the transforming effect of wild-type c-Myc in REF cells, possibly by heterodimerization with all available Max protein, leaving virtually no Max to form active heterodimers with wild-type c-Myc. Both of these *trans*-dominant mutants reinforce the concept that DNA binding by the c-Myc/Max heterodimer is necessary for c-Myc-induced malignant transformation. Mutations in the HLH or ZIP regions result in proteins that are unable to transform REF cells [16]. In contrast to the *trans*-dominant mutants, these proteins lack the ability to dimerize and are therefore unable to form inactive dimers to affect transformation by wild-type c-Myc.

At the molecular level, a straightforward consequence of specific DNA binding by c-Myc and Max is the tethering of these factors to DNA, allowing the remainder of the protein to exert an influence on nearby factors. In such a manner, the TADs of transcriptional activators are believed in some way to participate in the formation of the basal transcriptional complex at the promoter region of a gene, thus stimulating transcription of that gene [reviewed in 46,47]. Preliminary evidence has been presented that c-Myc/Max heterodimer is capable of such transcriptional activation (R.N. Eisenman, personal communication; L.M.S. Resar and C.V. Dang, unpublished).

It is somewhat less straightforward to consider how the c-Myc/Max heterodimer might affect the structure of the DNA on which it is bound. Recent work has shown that the Fos and Jun transcriptional activator proteins are able to bend DNA upon binding to their specific nucleotide recognition sequences [48]. It has been suggested that differential bending by these proteins may be responsible for their distinct biological effects in vivo. The potential significance of DNA bending is that protein-induced alteration in DNA conformation may facilitate initiation of transcription by bringing proteins bound to an upstream regulatory element in closer proximity to the transcription apparatus at the promoter region. Indeed, stretches of intrinsically bent DNA have been shown to functionally replace

sites of transcription factor-induced bending and to promote transcription in the absence of these factors [49]. Alternatively, the energy used to induce bending may increase the likelihood of unwinding the double helix, thereby facilitating the initiation of transcription [49,50].

Homo- and heterodimeric complexes of c-Myc and Max are able to cause increased DNA flexure as measured by circular permutation analysis [50a]. A mathematical derivation reveals that the angle of flexure induced by both homodimeric protein complexes is similar, approximately 53° from the horizontal. Since this technique does not permit determination of the orientation of bending, phasing analysis was used to determine whether different dimers induce bending in similar or different directions. Based on this analysis, Max and truncated c-Myc homodimers bend DNA in opposite orientations, whereas c-Myc/Max heterodimers cause a smaller bend in an orientation similar to that induced by Max homodimers. To address the possibility that the apparent opposite orientation of bending was the result of DNA unwinding by one of the proteins, the ability of c-Myc and Max homodimers to effect DNA unwinding was assayed. No specific unwinding caused by c-Myc or Max was demonstrable.

Nonspecific DNA binding by c-Myc

The c-Myc protein is distinct from related Myc family members because of the presence of a nonspecific DNA binding domain (NDBD). This domain is not essential for c-Myc transforming function; mutation or deletion of this region does not abrogate transformation. It may, however, appear to improve the kinetics of specific DNA binding by c-Myc through a mechanism of facilitated diffusion. The rationale and evidence for such a model is presented below.

Functional characterization of the nonspecific DNA binding domain

The NDBD was identified using peptide fragments of c-Myc produced in *E. coli* [22]. These c-Myc fragments were fused to staphylococcal Protein A, which served as an affinity tag, enabling rapid purification. A minimal c-Myc fragment representing amino acids 265–318 is sufficient to confer the ability to bind to random DNA sequences. This region contains four repeats that resemble a serine-proline-X-X motif (where X is any amino acid, commonly lysine) initially identified in the histone H4 protein, in which this motif mediates nonspecific DNA binding [21,51].

Proposed role of the nonspecific DNA binding domain in facilitated diffusion

The c-Myc NDBD is located in a region of c-Myc, which is not essential for transformation of REF cells [16]. Deletion of the NDBD, however, does

result in diminished efficiency of transformation. How does this second DNA binding domain contribute to the efficiency of c-Myc activity? In the *E. coli lac* repressor protein, the NDBD improves the kinetics of specific DNA binding through a mechanism of facilitated diffusion [52]. According to this model, nonspecific DNA binding serves to increase the local concentration of a protein around and on DNA, thereby enhancing the likelihood of encountering its specific chromosomal binding site(s). Data from similar investigations in prokaryotes suggest a model in which nonspecific DNA binding serves to promote a 'sliding and hopping' function, whereby the protein binds to a DNA molecule, sliding along the double helix and hopping to an adjacent DNA molecule until a specific high-affinity site is encountered [52]. Such a model would suggest that a high level of c-Myc expression could compensate for a deletion of NDBD function, but this has not been evaluated. Only indirect data exist for this proposed role for nonspecific DNA binding by c-Myc [25].

Potential role of phosphorylation in the regulation of DNA binding

It is well understood that, in vivo, c-Myc is a phosphoprotein and that in vitro, c-Myc can be phosphorylated by casein kinase II [53]. However, the biological effect of phosphorylation of c-Myc remains unclear. Provocative data concerning DNA binding by Max and by c-Myc/Max heterodimer raises the possibility that phosphorylation status may be important to DNA binding activity in vivo. Unphosphorylated Max homodimers bind to DNA readily. However, Max homodimers phosphorylated by casein kinase II are unable to bind to DNA [54]. Such phosphorylation does not appear to affect DNA binding by a c-Myc/Max heterodimer. The biological implication of modulation by phosphorylation is not clear, but this complex alteration of DNA binding properties by phosphorylation might provide a mechanism for regulation of DNA binding by c-Myc heterodimers and related proteins.

Summary

The c-Myc protein is a potential activator of transcription, with the ability to bind in a heterodimer form with Max to DNA sequences containing the core hexanucleotide sequence CAC(G/A)TG. These properties are shared with L-Myc, a homologous oncoprotein expressed in small cell lung carcinoma cells; with N-Myc, expressed in neuroblastoma cells; and with avian v-Myc, the c-Myc homolog expressed by a chicken retrovirus. The c-Myc, and probably v-Myc, proteins also have nonspecific DNA binding function, which may improve the kinetics of specific DNA binding. Curiously, this domain appears not to be conserved in L-Myc or N-Myc [22].

The data that have accumulated to date are consistent with a model in

which a c-Myc/Max heterodimer positively regulates the transcription of growth-related genes, with Max homodimer functioning as a negative regulator of the same genes (Fig. 4) [55]. Max is expressed constitutively at low levels, whereas c-Myc is expressed at low levels in quiescent cells, but high levels of c-Myc are induced by mitogenic stimulation [56]. Thus, in proliferating cells c-Myc/Max heterodimers might bind to the regulatory elements of growth-related genes, where the c-Myc TAD might stimulate transcription. Conversely, in quiescent cells with little c-Myc present, Max homodimers might predominate. They might bind to exactly the same regulatory elements, but due to the apparent absence of a TAD in Max [36], transcription might be repressed. Validation of this model will require the demonstration of clear regulation of a physiological promoter of a growth-related gene by c-Myc/Max.

Although it is widely believed that Myc proteins function as transcriptional activators, this hypothesis has only been conclusively supported recently [57,58]. A theory that c-Myc plays a role in DNA replication is not as well substantiated at this point. It is even possible that Myc might be involved in both transcription and replication. Although the function of these fascinating proteins has been enigmatic for a decade, the rate of

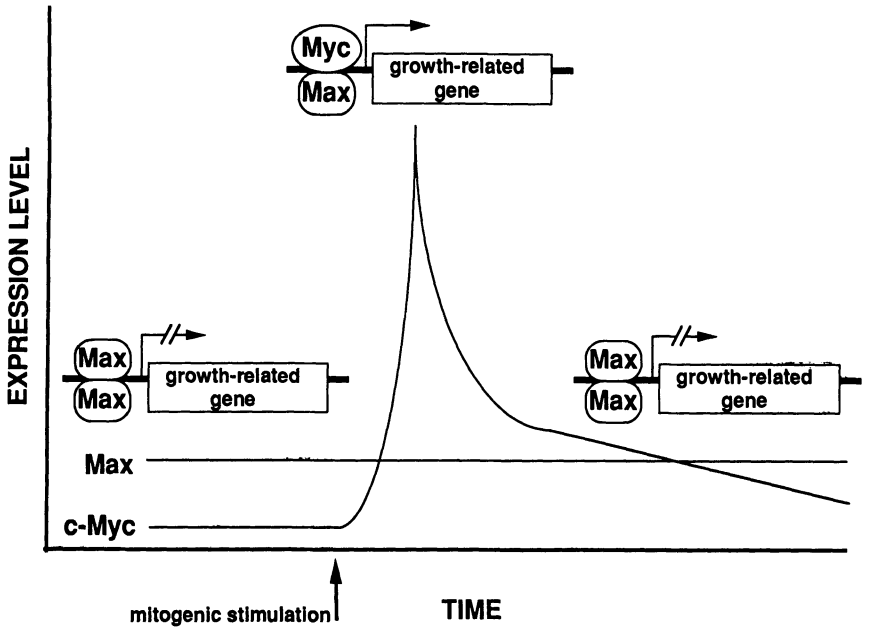

Figure 4. Proposed model of activity of c-Myc/Max heterodimers and Max homodimers. The graph depicts constant expression levels of Max and low levels of c-Myc in quiescent cells induced by mitogenic stimulus to high level c-Myc expression, gradually decreasing with time. The insets depict binding by c-Myc/Max heterodimer and Max homodimer to DNA regulatory elements of growth-related genes.

progress in our understanding of Myc function is accelerating. Such progress will undoubtedly lead to a deeper appreciation of this protein, which lies at the crossroads of cellular proliferation and oncogenesis.

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References

1. Duesberg PH, Bister K, Vogt PK: The RNA of avian acute leukemia virus MC29, Proc Natl Acad Sci USA 74:4320–4324, 1977.
2. Walker CW, Boom JDG, Marsh AG: First non-vertebrate member of the c-*myc* family is seasonally expressed in an invertebrate testis. Oncogene, in press, 1992.
3. Dalla Favera R, Bregni M, Erikson J, Patterson D, Gallo RC, Croce CM: Human c-*myc* oncogene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci USA 79:7824–7827, 1982.
4. Lee WM, Schwab M, Westaway D, Varmus HE: Augmented expression of normal c-*myc* is sufficient for cotransformation of rat embryo cells with a mutant ras gene. Mol Cell Biol 5:3345–3356, 1985.
5. Land H, Parada LF, Weinberg RA: Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304:596–602, 1983.
6. Adams JM, Harris AW, Pinkert CA, Corcoran LM, Alexander WS, Cory S, Palmiter RD, Brinster RL: The c-*myc* oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature 318:533–538, 1985.
7. Knight KL, Spiker-Polet H, Kazdin DS, Oi VT: Transgenic rabbits with lymphocytic leukemia induced by the c-*myc* oncogene fused with the immunoglobulin heavy chain enhancer. Proc Natl Acad Sci USA 85:3130–3134, 1988.
8. Taub R, Kirsch I, Morton C, Lenoir G, Swan D, Tronick S, Aaronson S, Leder P: Translocation of the c-*myc* gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci USA 79:7837–7841, 1982.
9. DePinho RA, Schreiber-Agus N, Alt FW: *myc* family oncogenes in the development of normal and neoplastic cells. Adv Cancer Res 57:1–46, 1991.
10. Hann SR, King MW, Bentley DL, Anderson CW, Eisenman RN: A non-AUG translational initiation in c-*myc* exon 1 generates an N-terminally distinct protein whose synthesis is disrupted in Burkitt's lymphomas. Cell 52:185–195, 1988.
11. Collum RG, Alt FW: Are Myc proteins transcription factors? Cancer Cells 2:69–75, 1990.
12. Luscher B, Eisenman RN: New light on Myc and Myb. Part 1. Myc. Genes Dev 4:2025–2035, 1990.
13. Murre C, McCaw PS, Baltimore D: A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and Myc proteins. Cell 56:777–783, 1989.

14. Prendergast GC, Ziff EB: Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. Science 251:186–189, 1991.
15. Blackwell TK, Kretzner L, Blackwood EM, Eisenman RN, Weintraub H: Sequence-specific DNA binding by the c-Myc protein. Science 250:1149–1151, 1990.
16. Stone J, de Lange T, Ramsay G, Jakobovits E, Bishop JM, Varmus H, Lee W: Definition of regions in human c-Myc that are involved in transformation and nuclear localization. Mol Cell Biol 7:1697–1709, 1987.
17. Schwab M, Alitalo K, Klempnauer KH, Varmus HE, Bishop JM, Gilbert F, Brodeur G, Goldstein M, Trent J: Amplified DNA with limited homology to *myc* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 305:245–248, 1983.
18. Kohl NE, Kanda N, Schreck RR, Bruns G, Latt SA, Gilbert F, Alt FW: Transposition and amplification of oncogene-related sequences in human neuroblastomas. Cell 35:359–367, 1983.
19. Nau MM, Brooks BJ, Battey J, Sausville E, Gazdar AF, Kirsch IR, McBride OW, Bertness V, Hollis GF, Minna JD: L-*myc*, a new *myc*-related gene amplified and expressed in human small cell lung cancer. Nature 318:69–73, 1985.
20. Blackwood EM, Eisenman RN: Max: A helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science 251:1211–1217, 1991.
21. Suzuki M: SPXX, a frequent sequence motif in gene regulatory proteins. J Mol Biol 207:61–84, 1989.
22. Dang CV, van Dam H, Buckmire M, Lee WM: DNA-binding domain of human c-Myc produced in *Escherichia coli*. Mol Cell Biol 9:2477–2486, 1989.
23. Kato GJ, Dang CV: Function of the c-Myc oncoprotein. FASEB J 6:3065–3072, 1992.
24. Sarid J, Halazonetis TD, Murphy W, Leder P: Evolutionarily conserved regions of the human c-Myc protein can be uncoupled from transforming activity. Proc Natl Acad Sci USA 84:170–173, 1987.
25. Kato GJ, Barrett J, Villa Garcia M, Dang CV: An amino-terminal c-Myc domain required for neoplastic transformation activates transcription. Mol Cell Biol 10:5914–5920, 1990.
26. Dang CV, Lee WM: Identification of the human c-Myc protein nuclear translocation signal. Mol Cell Biol 8:4048–4054, 1988.
27. Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB: Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58:537–544, 1989.
28. Clarke MF, Kukowska Latallo JF, Westin E, Smith M, Prochownik EV: Constitutive expression of a c-*myb* cDNA blocks Friend murine erythroleukemia cell differentiation. Mol Cell Biol 8:884–892, 1988.
29. Anthony-Cahil SJ, Benfield PA, Fairman R, Wasserman ZR, Brenner SL, Stafford III WF, Altenbach C, Hubbell WL, DeGrado WF: Molecular characterization of helix-loop-helix peptides. Science 255:979–983, 1992.
30. Voronova A, Baltimore D: Mutations that disrupt DNA binding and dimer formation in the E47 helix-loop-helix protein map to distinct domains. Proc Natl Acad Sci USA 87:4722–4726, 1990.
31. Beckman H, Su LK, Kadesch T: TFE3: A helix-loop-helix protein that activates transcription through the immunoglobulin enhancer muE3 motif. Genes Dev 4:167–179, 1990.
32. Gregor PD, Sawadogo M, Roeder RG: The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. Genes Dev 4:1730–1740, 1990.
33. Hu YF, Luscher B, Admon A, Mermod N, Tjian R: Transcription factor AP-4 contains multiple dimerization domains that regulate dimer specificity. Genes Dev 4:1741–1752, 1990.
34. Landschulz WH, Johnson PF, McKnight SL: The leucine zipper: A hypothetical structure

common to a new class of DNA binding proteins. Science 240:1759–1764, 1988.
35. Beckmann H, Kadesch T: The leucine zipper of TFE3 dictates helix-loop-helix dimerization specificity. Genes Dev 5:1057–1066, 1991.
36. Kato GJ, Lee WM, Chen LL, Dang CV: Max: Functional domains and interaction with c-Myc. Genes Dev 6:81–92, 1992.
37. Dang CV, Barrett J, Villa Garcia M, Resar LM, Kato GJ, Fearon ER: Intracellular leucine zipper interactions suggest c-Myc hetero-oligomerization. Mol Cell Biol 11:954–962, 1991.
38. Prendergast GC, Lawe D, Ziff EB: Association of Myn, the murine homolog of Max, with c-Myc stimulates methylation-sensitive DNA binding and *ras* cotransformation. Cell 65:395–407, 1991.
39. Wenzel A, Czielpluch C, Hamann U, Schurmann J, Schwab M: The N-Myc oncoprotein is associated in vivo with the phosphoprotein Max (p20/22) in human neuroblastoma cells. EMBO J 10:3703–3712, 1991.
40. Dang CV, Dolde C, Gillison ML, Kato GJ: Discrimination between related DNA sites by a single amino acid residue of Myc-related basic-helix-loop-helix proteins. Proc Natl Acad Sci USA 89:599–602, 1992.
41. Cai M, Davis RW: Yeast centromere binding protein CBF1, of the helix-loop-helix protein family, is required for chromosome stability and methionine prototrophy. Cell 61:437–446, 1990.
42. Halazonetis TD, Kandil AN: Determination of the c-MYC DNA-binding site. Proc Natl Acad Sci USA 88:6162–6166, 1991.
43. Papoulas O, Williams NG, Kingston RE: DNA-binding activities of c-Myc purified from eukaryotic cells. J Biol Chem, in press, 1992.
44. Eilers M, Schirm S, Bishop JM: The MYC protein activates transcription of the alpha-prothymosin gene. EMBO J 10:133–141, 1991.
45. Dang CV, McGuire M, Buckmire M, Lee WM: Involvement of the 'leucine zipper' region in the oligomerization and transforming activity of human c-Myc protein. Nature 337:664–666, 1989.
45a. Sawyers CL, Callahan W, Witte ON: Dominant negative myc blocks transformation by *ABL* oncogenes. Cell 70:901–910, 1992.
46. Mitchell PJ, Tjian R: Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245:371–378, 1989.
47. Mermelstein FH, Flores O, Reinberg D: Initiation of transcription by RNA polymerase II. Biochim Biophys Acta 1009:1–10, 1989.
48. Kerppola TK, Curran T: Fos-Jun heterodimers and Jun homodimers bend DNA in opposite orientations: Implications for transcription factor cooperativity. Cell 66:317–326, 1991.
49. Gartenberg MR, Crothers DM: Synthetic DNA bending sequences increase the rate of in vitro transcription initiation at the *Escherichia coli* lac promoter. J Mol Biol 219:217–230, 1991.
50. Zinkel SS, Crothers DM: Catabolite activator protein-induced DNA bending in transcription initiation. J Mol Biol 219:201–215, 1991.
50a. Wechsler DS, Dang CV: Opposite orientations of DNA bending by c-Myc and Max. Proc Natl Acad Sci USA 89:7635–7639, 1992.
51. Suzuki M: SPKK, a new nucleic acid-binding unit of protein found in histone. EMBO J 8:797–804, 1989.
52. von Hippel PH, Berg OG: Facilitated target location in biological systems. J Biol Chem 264:675–678, 1989.
53. Luscher B, Kuenzel EA, Krebs EG, Eisenman RN: A phosphorylation site located in the NH_2-terminal domain of c-Myc increases transactivation of gene expression. EMBO J 8:1111–1119, 1989.
54. Berberich SJ, Cole MD: Casein kinase II inhibits the DNA-binding activity of Max homodimers but not Myc/Max heterodimers. Genes Dev 6:166–176, 1992.
55. Cole MD: Myc meets its Max. Cell 65:715–716, 1991.

56. Blackwood EM, Luscher B, Eisenman RN: Myc and Max associate in vivo. Genes Dev 6:71–80, 1992.
57. Amati B, Dalton S, Brooks MW, Littlewood TD, Evan GI, Land H: Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max. Nature 359:423–426, 1992.
58. Kretzner L, Blackwood EM, Eisenman RN: Myc and Max proteins possess distinct transcriptional activities. Nature 359:426–429, 1992.

17. Normal and malignant growth control by p53

Cathy A. Finlay

Introduction

The role of the p53 protein in the growth of both the normal and the transformed cell has been the focus of investigation since the discovery of p53 12 years ago. Although the function of this protein is not yet known, p53 appears to be a critical protein involved in the regulation of cell growth. Once classified as a dominant oncogene [1–3], it has recently become clear that only mutant forms of p53 can contribute to cellular transformation [4–6]. In contrast, overexpression of the wild-type p53 protein suppresses the formation of transformed cells [7,8] and, in addition, inhibits the growth of tumor cells [9–13]. These observations have resulted in the redefinition of the role of p53 to that of a recessive oncogene or a tumor suppressor gene [reviewed in 14,15]. Numerous studies conducted in the past few years have shown that alterations (deletions, rearrangements, missense mutations) in the p53 gene occur frequently (25–85% of the time) in quite a wide variety of human tumors [16–40]. Thus, mutations at the p53 locus are, at present, the most common genetic change known to occur in human cancer. Do certain mutations appear to be selected for in human tumors? What are the known activities and phenotypes of the p53 protein in the normal cell, and what is the effect of mutation on the known p53 properties? What is the role, if any, of the overexpressed mutant p53 proteins in the tumor cell?

From oncogene to tumor suppressor gene

p53 is a cellular protein expressed at low levels in normal cells and tissues [41–44]. In most nontransformed cells, the p53 protein (so named because the migration of this protein on SDS-polyacrylamide gels indicated a molecular weight of 53,000 Da) has a short half-life, 20–30 minutes, depending on the cell type [45,46]. p53 was discovered as a protein that coimmunoprecipitated with the transforming protein of simian virus 40 (SV40), the large T antigen, in SV40 transformed cells following immunoprecipitation with anti-sera from SV40 tumor bearing animals [47,48]. In SV40 trans-

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formed cells, p53 is found in oligomeric protein complexes with large T antigen, and the levels of p53 are 100-fold higher than that observed in normal cells; the half-life of p53 is correspondingly extended (from 20–30 minutes to over 24 hours) [45,46]. p53 also forms a stable complex with one of the transforming proteins of adenovirus type 5, the E1b 55K protein [49]. The fact that the transforming proteins from two different viruses bound p53 suggested that p53 plays a role in the transformation of cells by these viruses and that this protein was critical in the regulation of cell growth. In accord with this hypothesis, a positive role for p53 in the cell cycle was demonstrated by Mercer and coworkers [50,51], who showed that p53 expression is essential for cells to enter the cell cycle; the microinjection of a monoclonal antibody specific for p53 into Balb/c 3T3 cells within the first 4 hours of serum stimulation blocked the entry of these cells into DNA synthesis. Numerous studies, which examined p53 expression in a wide variety of transformed cells, tumors, and tumor-derived cell lines, soon demonstrated that p53 levels were frequently elevated in the transformed cell [41–44,52,53]. Thus, overexpression of p53 was correlated with the transformed phenotype.

To determine if elevated levels of p53 could effect changes in the phenotype of the normal cell, murine p53 genomic or cDNA clones were overexpressed in rodent cells. High levels of p53 resulted in the immortalization of primary rat chondrocytes [1] or rat embryo fibroblasts (REFs) [54,55], and when assayed in conjunction with an activated *ras* gene, in the full transformation of primary rat fibroblasts [2,3]. High levels of p53 expression also enhanced the transformation frequency of primary rodent cells by the SV40 large T antigen [56]. In addition, when p53 was overexpressed in an immortalized rodent cell line (Rat 1), this resulted in the increased tumorigenicity of these cells [57]. Overexpression of p53 in a murine bladder carcinoma cell line increased the metastatic capacity of these cells [58]. Similar results were observed when p53 was introduced into an Abelson murine leukemia virus transformed cell line (L12 cells) that did not express any endogenous p53; the L12 cells were converted from a cell line that formed tumors that regressed to one that formed progressively growing, eventually lethal tumors [59,60]. Thus, utilizing a variety of assay systems, p53 acted as a oncogene.

Some p53 clones, however, did not possess transforming properties in these assays. For example, similar experiments conducted utilizing a murine p53 cDNA cloned from the F9 teratocarcinoma cell line did not show p53 to have transforming activity [61]; i.e., this clone did not cooperate with an activated *ras* gene to transform primary REFs [4,61]. The situation was clarified when the sequences of the transforming p53 genomic and cDNA clones were compared with the F9 murine cDNA sequence; each transforming sequence differed from the F9 sequence by a single (different) amino acid [4]. Detailed studies soon demonstrated that a single nucleotide change resulting in a single amino acid change (missense mutation) did activate p53 for transformation [5,6]. The activating mutations are located in

a region spanning over 25% of the p53 gene (amino acids 130–240 out of 390) and result in stabilization of the p53 protein with an elevation of p53 levels [4,6]. The diversity of mutations that activate p53 has led to the hypothesis that these mutations are dominant loss-of-function mutations and that one mechanism by which p53 can act to transform cells in tissue culture is to form oligomeric protein complexes with the wild-type endogenous rat p53 and inactivate a wild-type regulatory function [62]. Complexes between wild-type and mutant p53 proteins have been noted by several investigators [6,7,54], and levels of mutant p53 approximately 100-fold higher than that of endogenous p53 are necessary for efficient transformation [5]. In light of the above observations, the purpose of the SV40 large T antigen/p53 and the adenovirus E1B/p53 complexes may be to inactivate the regulatory actions of p53, thus providing one of the common mechanism(s) by which a virus can control the cell cycle of the infected cell. Of interest is the recent observation that wild-type human p53 can interact in vitro with another viral protein, the E6 protein from human papilloma viruses 16 and 18 [63]. In this instance, however, p53 is destabilized through a ubiquitin-mediated mechanism [64]. Complexes are also formed between the retinoblastoma (RB) tumor suppressor gene product and transforming proteins from the same three viruses, the SV40 large T antigen, the adenovirus Type 5 E1a protein, and the human papilloma virus Type 16 E7 proteins [65–67].

Insights into the biological activities of wild-type p53 were provided by in vitro evidence that p53 could act as a suppressor of cell transformation. Indeed, when transfected with two cooperating oncogenes such as *ras* and adenovirus type 5 E1a or *ras* plus mutant p53, wild-type p53 suppressed the formation of transformed foci in primary rat cells [7,8]. Cell lines (13/13) derived from *ras* plus E1a plus wild-type p53 transfections either did not express wild-type p53 or expressed mutant forms of the protein [7]. In contrast, mutant p53 proteins did not suppress transformed cell growth [7,8]. In fact, mutant p53 enhanced the transformation of REFs by *ras* plus E1a in these assays and was expressed in all (15/15) of the cell lines examined [7]. A summary of mutant and wild-type p53 activities in transforming assays is presented in Table 1.

Although the above studies demonstrated that wild-type p53 could suppress transformed cell growth in vitro, additional support for the role of p53 as a tumor suppressor was provided by evidence that mutations in p53

Table 1. p53 activity in transformation assays

Assay	Wild-type p53	Mutant p53
Immortalization of rat cells [1,54,55]	–	+
Transformation of REF in cooperation with *ras* [2–4]	–	+
Enhanced tumorigenicity of rodent cells [57–60]	–	+
Suppression of transformation of REF by two cooperating oncogenes [7,8]	+	–

occurred in the development of tumors in vivo. Elegant studies characterizing the role of p53 in Friend virus induced murine erythroleukemias had previously shown that p53 was either deleted or overexpressed in these tumors; the overexpressed p53 was mutant, confirming that the inactivation of p53 occurred in tumor development in vivo [68–72]. Overexpression of a p53 clone derived from one of these murine erythroleukemia cell lines resulted in immortalization of primary REFs [54]. The laboratory of Bert Vogelstein extended these observations into human tumors with meticulous studies on p53 in human colorectal cancer; in 85% of colorectal cancers, one allele of p53 (on human chromosome 17p) has typically been deleted, while the remaining allele carries a missense mutation [18,22,73]. As demonstrated for mutant murine p53 clones, the mutant human p53 clones derived from colorectal tumors were transforming in cooperation with an activated *ras* gene, although to differing degrees [74]. These data are consistent with a model [22,74] for tumor progression in which a precancerous cell possessing a missense mutation in p53 has a growth advantage over cells possessing two wild-type alleles (through a dominant negative mechanism). The increased proliferative capacity of these cells would result in more cell divisions, thus increasing the probability of a second mutation (allelic loss) occurring at the wild-type allele. The resultant cell with a single mutant p53 gene (and no wild-type allele) would then be fully transformed.

Wild type p53 effects on transformed cell growth

If the p53 protein as a tumor suppressor, then the expression of p53 in tumor cell lines should effect changes in the growth properties of tumor cells (for example, the ability to enter DNA synthesis or growth in soft agar). To address this question, wild-type p53 was introduced into a variety of transformed cell lines [9–13]. In most instances, wild-type p53 expression was incompatible with transformed cell growth, i.e., following cotransfection with a selectable marker and cDNA or genomic clones expressing wild-type p53 (often under strong promoter enhancer regions [9,10]) a reduction in the plating efficiency of the cells was observed and no colonies were obtained that expressed the wild-type protein [9–11]. This result was observed whether the recipient cells were expressing either no endogenous p53 [10,11] or mutant endogenous p53 [9–11]. In contrast, transfection of wild-type p53 into normal primary REFs did not alter the plating efficiency of these cells [7,8,11], and exogenous p53 expression was observed in clones derived from these transfections [11]. Thus p53 overexpression was selectively incompatible with transformed cell growth.

The technical problems involved in determining the effects of p53 on transformed cell growth in vitro were circumvented utilizing a variety of experimental methods. First, it was recently discovered that a murine mutant clone, $p53^{val135}$ (possessing a valine instead of an alanine at amino

acid 135), had a temperature-sensitive phenotype [75]. At 32°C, the protein is predominantly in a wild-type conformation (assayed by recognition by a murine-specific monoclonal antibody, PAb 240 [76]), does not cooperate with an activated *ras* gene, and can suppress the development of transformed foci. At 39°C, the protein is primarily in a mutant conformation, assayed by recognition by monoclonal antibody PAb 240 [77], and does act like an oncogene. A *ras* plus $p53^{val135}$ transformed cell line, when shifted to 32°C, reversibly arrests in the G_1 phase of the cell cycle. This growth arrest occurs concomitantly with the translocation of $p53^{val135}$ protein from the cytoplasm into the nucleus [78–80], suggesting that p53 must be in the nucleus to exert growth regulatory effects. In a novel observation, Yonish-Rouach and co-workers [81] noted that at 32°C, expression of $p53^{val135}$ (wild-type conformation) resulted in the programmed cell death (apoptosis) of a murine myeloid leukemic cell line. Second, the negative effect of wild-type p53 on the growth of a human glioblastoma cell line, T98G, was examined by controlling p53 expression with a hormone-inducible promoter (the mouse mammary tumor virus promoter); induction of wild-type p53 resulted in the inhibition of cell growth with the cells arrested in the G_1 phase of the cell cycle [12]. Third, the inability of cells overexpressing wild-type p53 to enter DNA synthesis was also demonstrated by analyzing cells within 48 hours after transfection; growth inhibition (measured as a decline in the population of cells synthesizing DNA) was observed in Saos-2 cells (a human osteosarcoma cell line that has deleted p53 [82] and in a colorectal carcinoma cell line (expressing low levels of endogenous wild-type p53) [9,10]. This inhibition was not observed, however, in an adenoma cell line (derived from a benign colorectal tumor), again showing this effect was specific to carcinoma cells [9]. Fourth, Saos-2 cell lines expressing a single copy of human wild-type p53 were generated by retroviral infection [13]. Although the Saos-2 cells expressing wild-type p53 continued to proliferate, the doubling time of the cells was increased, and the cells no longer grew in soft agar or formed tumors in nude mice [13]. In addition, clonal cell lines expressing both a human wild-type and a human mutant (at amino acid 273) p53 [13] were obtained. Although Saos-2 cells expressing the 273 mutant grew in soft agar, formed tumors in nude mice, and also possessed a limited growth advantage over the parent cell (increased saturation density), cell

Table 2. Effects of wild-type p53 expression on transformed cell growth

Recipient cell	Effect
Tumor cells [9–11]	Reduction in plating efficiency
Transformed rat cells [75,78–80]	
Human glioblastoma cell line [12]	Growth arrest
Human osteosarcoma cell line [10]	
Human colorectal carcinoma cell line [9]	
Murine myeloid leukemia cell line [81]	Apoptosis
Human osteosarcoma cell line [13]	Reduction in tumorigenicity

lines expressing both the mutant and wild-type protein had the phenotype of cell lines expressing wild-type p53; that is, these cells no longer grew in soft agar and no longer formed tumors in nude mice [13]. The extended half-life of the mutant protein resulted in mutant protein levels 10-fold higher than that of the wild-type protein; therefore, in the presence of this mutant the phenotype of the wild-type protein is dominant. The effects of wild-type p53 on transformed cell growth are summarized in Table 2.

p53 mutations in human cancer

What types of p53 mutations are found in human tumors? Since the initial observations on p53 mutations in human colorectal cancer 2 years ago, mutational analyses of the human p53 gene (located on the small arm of human chromosome 17 (17p) [83–85]) have been presented for over 250 cell lines, tumor-derived cell lines, and primary tumors and xenografts. Since p53 mutations are common in cell lines [4] and in some instances occur at a higher frequency than observed in the primary tumor cell [32], this review will focus on p53 mutations found in human tumors. These data may be limited by the fact that investigators often do not sequence the entire p53 gene, since mutations have been shown to occur less frequently in the amino and carboxy terminus [18,29,73]. To date, p53 mutations (rearrangements, deletions, and point mutations resulting in missense or frameshift mutations or termination codons) have been found in tumors of the anus [16], bone [35,36], bladder [17], brain [18], breast [19–21], colon [22], esophagus [23,24], stomach [25], liver [27,28], lung [29–31,86], lymphoid system [32,33], ovary [37], and prostate [38], demonstrating that p53 must play a critical role in the growth regulation of many different tissues. The vast majority of these mutations are missense mutations (73%), suggesting mutant p53 is beneficial to cell growth. A graphic compilation of 148 missense mutations found in human tumors is presented in Fig. 1. A similar tabulation of p53 mutations in human cancers has recently been provided by Hollstein et al. [87].

There are several points to be made about the pattern of p53 mutations in human tumors (Fig. 1). First, the missense mutations do not occur randomly; the mutations are clustered in four regions of the p53 protein that are highly conserved from rainbow trout to humans (Domains II–V) (Table 3), indicating these regions are of functional importance. Second, there are mutational 'hot spots' [18] at amino acids 175 (17/148), 248–249 (25/148), 273 (9/148), and 282 (8/148). Mutations at these amino acids account for approximately 40% of the total missense mutations observed in human tumors. Third, different 'hot spots' are present in different tissues. For example, mutations at amino acid 175 have been found repeatedly in colorectal carcinomas (5/25) [22] and in Burkitt's lymphoma (3/10) [32], and have been observed at least once in breast carcinoma [19], glioblastoma

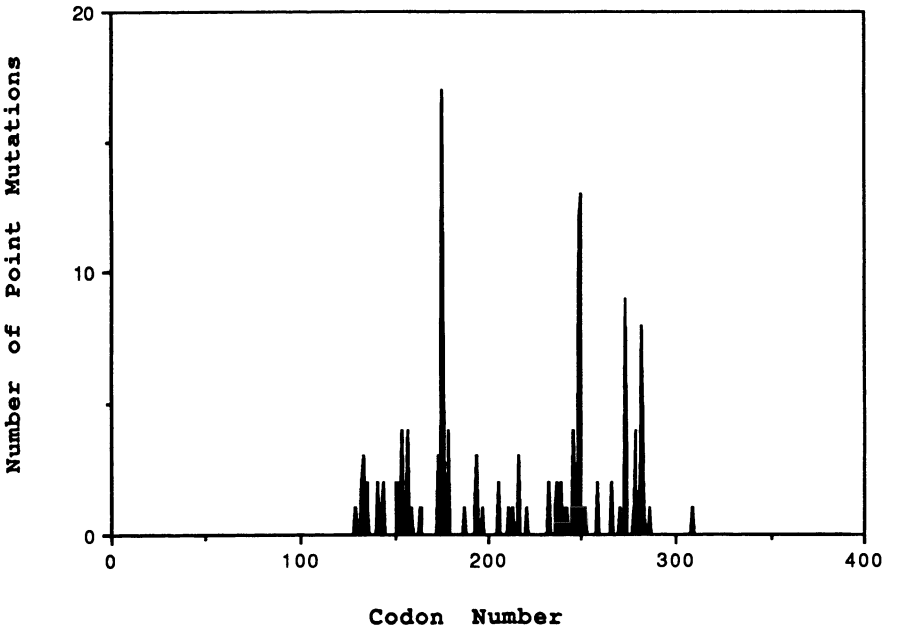

Figure 1. The distribution of p53 missense mutations in human tumors. The horizontal axis represents the 393 codons of the human p53 gene from the amino terminus (codon 1) to the carboxy terminus (codon 393). The vertical axis represents the number of total missense mutations observed at a specific codon. These data are a compilation of p53 mutations found in 148 primary tumors or xenografts [16–32,34,36–40,86].

[18], and an esophageal squamous cell carcinoma [23]. An exception is lung carcinomas, where a mutation at 175 has not yet been observed. Mutations at amino acid 249 have been found in almost 50% of the hepatocellular carcinomas examined from high-incidence areas of Qidong, China [28] and southern Africa [27]. The reason for the different frequencies of mutations in different tissues is not yet known. Tissue-specific 'hot spots' could reflect differences in the intracellular environment or the mutagens present in a given tissue. For example, Hollstein et al. [87] have noted that mutations resulting from G:C to T:A transversions occur commonly in hepatocellular carcinomas and in lung carcinomas, perhaps reflecting exposure to specific carcinogens (Aflatoxin B1 in the case of hepatocellular carcinoma, for example). In contrast, missense mutations resulting from transitions (G:C to A:T) at CpG dinucleotides are most common in colon cancer and leukemias and lymphomas [87]. An alternate explanation is that point mutations occur randomly in the p53 gene in each tissue, but different mutant p53 proteins contribute a growth advantage to different cells (and are selected for) in a tissue-specific manner.

The presence of a mutant p53 allele is often correlated with the loss of the remaining wild-type p53 allele. For example, this has been observed in colon

and lung carcinomas and in lymphomas and leukemias, suggesting the mutant phenotype is recessive in the presence of the wild-type allele [22,29,32]. Detailed analysis has shown that a mutant and wild-type allele co-exist in colon carcinomas less than 30% of the time (when compared to all carcinomas expressing 2 alleles) [22]. The presence of a mutant allele is strongly correlated with the loss of the remaining allele; 91% of one allele carcinomas possess a mutant p53 protein. Thus, at least in this tumor, there appears to be a strong selection for the inactivation of both alleles in quick succession. In breast carcinomas, however, examination of p53 mutations and loss of heterozygosity (LOH) has demonstrated that the two events are not correlated, i.e. p53 mutations are found equally frequently in tumors with or without a wild-type allele [20]. Conversely, approximately 40% of the tumors possessing a single allele are found to express a wild-type p53 [20]. It will be of interest to determine if the levels of mutant p53 expressed in breast tumors expressing both a wild-type and a mutant p53 are higher than those observed in breast tumors expressing only a mutant allele, thus conferring a notable growth advantage even in the presence of the wild-type allele.

Germline mutations in p53

The predominance of p53 mutations found in human cancer occur as somatic mutations in the development of the tumor. Do germline mutations in p53 contribute to tumor formation in humans? In an animal model system, transgenic mice carrying a mutant p53 gene (in the presence of two endogenous wild-type alleles) develop a variety of tumors (lung adenocarcinomas, lymphomas, and osteosarcomas) following a long latent period, suggesting that mutant p53 can predispose to tumor formation [88]. This question was addressed by examining the p53 gene in individuals from families affected by the Li-Fraumeni syndrome (a rare autosomal dominant syndrome characterized by diverse neoplasms at multiple sites [89,90]). Germline mutations in p53 have been found in individuals from six Li-Fraumeni families [26,91]. To date, all of these mutations in p53 have been found in conserved region IV (Table 3). The wild-type allele (present in the normal tissue from these people) is generally deleted in the tumors, while

Table 3. Highly conserved domains of the p53 protein [101]

Domain	Amino acid residues
I	13–19
II	117–142
III	171–181
IV	236–258
V	270–286

Table 4. Properties of p53 human wild-type and mutant proteins

Property	WT	143[a]	175	248	273	281
Relative transformation frequency [74]	0	2	22	ND[b]	8	4
Transformation suppression [9,10,13,92]	+	−	−	−	−	−
Conformational alteration [74, R. Quartin and A.J. Levine, unpublished]	WT[c]	Mutant	Mutant	WT	WT	WT
Protein half-life (hours) [74]	0.3	1.5–2	3.6	ND	7	1.4
Transactivation of a test gene [95,96]	+	−	−	ND	+	ND
Binding to T antigen [93, R. Quartin and A.J. Levine, unpublished]	+	−(10%)	−	−	−	−

[a] Assayed in the absence of introns.
[b] ND = not determined.
[c] WT = wild type.

the mutant allele is retained [26]. In addition, another germline mutation was found at amino acid 242 in a young patient with an intracranial ependymoma [40]. Since individuals carrying germline mutations in p53 develop normally, this would suggest that the deregulating effects of mutant p53 in the presence of a wild-type allele are subtle. This may reflect the phenotype of p53 proteins with mutations in domain IV (Table 4). The prevalence of both somatic and germline mutations in this region (amino acids 242–258) (Fig. 1) indicates that this region of the protein is critical to p53 function.

The properties of mutant p53 proteins

The prevalence of p53 missense mutations in human tumors suggests that the elevated expression of a full-length mutant protein has been selected for in tumor development. Are the transforming activities of p53 mutants similar? Are there properties common to all mutants? To answer these questions, mutant p53 cDNA clones have been isolated from colorectal tumors and the mutant proteins have been characterized. The results from these studies are presented in Table 4. In all cases mutant p53 proteins have been found to be nonsuppressive in transformed cell growth assays, demonstrating the loss of the growth-inhibitory wild-type function [9,10,13,92]. In addition, the half-lives of the mutant proteins are long (generally several hours) [74] and the ability of mutant p53 proteins to complex with SV40 T antigen is reduced or eliminated [93,94; R. Quartin and A.J. Levine,

unpublished]. p53 proteins with mutations at amino acids 143 and 175 complex with the constitutively expressed member of the heat shock family, hsc 70, and are recognized by the mutant specific monoclonal antibody PAb240 [74; R. Quartin and A.J. Levine, unpublished]. p53 proteins with mutations at amino acids 248, 273, or 281, however, possess less altered conformations (are not recognized by PAb240 and do not bind to hsc 70 [74; R. Quartin and A.J. Levine, unpublished]). The transforming activities of these mutants are also not equivalent; on average, the 175 mutant reproducibly yields 2.5- to 6-hold more foci than the 273 or the 281 mutant, respectively, when assayed in conjunction with an activated *ras* gene [74]. The wild-type p53 protein and the 273 mutants can transactivate a test gene; however, the 143 and 175 mutants cannot [95,96]. In these transactivation assays, the carboxy terminus of p53 is replaced with the Gal-4 DNA binding domain; therefore, this assay masks deficiencies in DNA binding and oligomerization that may exist in those mutants that score as active in these assays. Indeed, the 273 mutant transactivates [96]; however, the ability of the mutant protein to bind DNA is strongly reduced [93].

Thus, different mutations alter different properties of p53. As the characterization of mutant p53 continues, the number of p53 mutant phenotypes will no doubt continue to increase. Different amino acid changes at the same amino acid residue could result in proteins with different characteristics. Perhaps, different mutants can influence the growth of the tumor cell and affect the prognosis of the cancer. If so, a greater understanding of the role of mutant p53 proteins and the proteins they interact with is critical for diagnosis and effective treatment of human cancer.

The results of numerous studies examining the ability of mutant p53 to mitigate the growth-suppressive effects of wild-type p53 have shown that, in most instances, the wild-type phenotype is dominant. Previous work has shown, however, that 100-fold or greater excess of a mutant protein may be necessary for sufficient transforming activity to be observed in tissue culture [5]. Therefore, the relative levels of mutant and wild-type p53 may determine the outcome of experiments, and the overexpression of wild-type p53 in many of the transfection experiments could have influenced the results. Alternatively, the phenotype of the mutant could determine how wild-type properties are affected in the heterozygous state. For example, Milner and Medcalf [97] have shown that human p53 clones with mutations at amino acids 151, 247, or 273, when cotranslated with the wild-type protein, can drive the wild-type protein into the mutant conformation, and hypothetically, interfere with normal function. Of particular interest is the observation that the human p53 mutant with a mutation at amino acid 248 (seen in Li-Fraumeni families [26]) does not alter the conformation of the wild-type protein [97]. Thus these data provide an explanation for the observation that the transforming activities of different mutants are not the same [74,98].

Critical to our understanding of p53 in malignant cell growth is the

knowledge of whether the mutant p53 proteins are advantageous to tumor cell growth. There are data that demonstrate mutations in p53 can result in a gain of a growth-promoting function in tumor cells. First, the expression of mutant p53 in murine L12 cells (which do not express p53 and can only form tumors that regress in nude mice) results in cells that are fully tumorigenic [59,60]. In addition, disruption of the nuclear localizaion signal eliminates this effect, suggesting that, at least for some mutants, positive growth effects are exerted in the nucleus [60]. Second, expression of the endogenous mutant p53 is necessary for the growth of methylcholanthrene transformed cells [99,100]. Third, minimal growth-promoting effects were also noted when mutant p53 was introduced into Saos-2 cells [13].

The function(s) of p53

Although mutations in p53 eliminate the ability of this protein to regulate transformed cell growth, the function(s) of wild-type p53 that is involved in normal cell growth regulation has yet to be defined. At present there are two hypotheses to explain the ability of p53 to control cell proliferation. First, p53 could act as a transactivator of gene expression, turning on or off the expression of genes involved in the cell cycle. The primary structure of p53 is compatible with the hypothesis that p53 is a transactivator. The structure of p53 can be separated into three domains: (1) an acidic, highly charged amino-terminal region (amino acids 1–75), followed by (2) an extended hydrophobic proline-rich region (amino acids 75–150), with (3) a highly charged basic carboxy-terminal domain (amino acids 275–390) [101]. The carboxy terminus possesses nuclear localization signals [60,102,103], and the oligomerization [104] and DNA binding domains [95]. Although early experiments showed that p53 could bind DNA in a nonspecific fashion [105–107], the results of recent studies demonstrate p53 can bind to DNA in a sequence-specific manner [93,108]. Mutations in p53 reduce the ability of p53 to bind DNA [93,107,108]. In 1990, direct support for the role of p53 as a transactivator of gene expression emerged from several laboratories [96,109,110]; when fused to a Gal-4 DNA binding domain, p53 activated the expression of reporter genes regulated by Gal-4 DNA binding sequences. This activity was mapped to the acidic amino terminus [96,110]. Murine and human mutants that bind to hsc 70 did not transactivate in this assay (Table 4) [95,109]. In transient assays, wild-type p53 expression has also resulted in both the activation [111] and the repression [112] of expression of reporter sequences from cellular promoters. It will be of interest to determine what promoter-enhancer regions contain the sequences specifically recognized by p53 and to demonstrate the activation or repression of these genes by p53. Second, p53 could be involved in the regulation of the initiation of DNA synthesis. The majority of evidence supporting this role for p53 comes from the association of p53 with the SV40 large T antigen. The SV40 large T

antigen is required for the initiation of viral DNA replication. Wild-type p53 can compete with DNA polymerase alpha for binding to T antigen [113], and wild-type, but not mutant, p53 expression can inhibit viral DNA replication in vivo and in vitro [114–117]. A simplistic explanation for these observations is that in the normal cell p53 binds a homologue(s) of T antigen involved in the initiation of cellular DNA synthesis. The formation of a complex with p53 could prevent the association of this protein with DNA polymerase alpha (or another critical cellular protein), negatively controlling cell growth. The inability of mutant p53 proteins to bind T antigen [93,94] suggests the p53 mutants are no longer capable of binding the appropriate cellular protein and regulating cell growth. In both of these models, a regulatory function of p53 has been lost. How could p53 mutants positively affect cell growth in the absence of a wild-type allele? Different p53 proteins could bind (with differing affinities) to cellular proteins (that may or may not associate with the wild-type p53 protein) involved in growth control. Characterization of p53-associated proteins [74] could thus provide insights into the role of mutant p53 in cell growth.

Conclusions

Since the acceptance of p53 as a recessive oncogene or tumor suppressor gene, there has been rapid progress in the characterization of the function(s) of wild-type p53 and the effect of mutation on p53 properties. The wild-type protein may control growth at several points during the cell cycle, interacting in succession with a variety of cellular proteins. Different mutations alter different properties of the protein and are likely to affect these interactions, and thus to alter cell growth. It is rapidly becoming clear that a minimum of several different classes of p53 mutants exist. As these data continue to accumulate, the p53 mutants associated with each tumor type may become more defined, providing greater insights into the carcinogens and/or repair mechanisms present in each tissue type. Whether the different mutants have growth-promoting properties in the normal and malignant cell is an important question yet to be answered. An understanding of the differential growth-promoting effects of mutant p53 expression could be invaluable in determining the proper diagnosis and most effective course of treatment of human cancers.

References

1. Jenkins JR, Rudge K, Currie GA: Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. Nature 312:651–654, 1984.
2. Parada LF, Land H, Weinberg RA, Wolf D, Rotter V: Cooperation between gene encoding p53 tumour antigen and *ras* in cellular transformation. Nature 312:649–651, 1984.

3. Eliyahu D, Raz A, Gruss P, Givol D, Oren M: Participation of p53 cellular tumor antigen in transformation of normal embryonic cells. Nature 312:646–649, 1984.
4. Finlay CA, Hinds PW, Tan T-H, Eliyahu D, Oren M, Levine AJ: Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. Mol Cell Biol 8:531–539, 1988.
5. Hinds P, Finlay C, Levine AJ: Mutation is required to activate the p53 gene for cooperation with the *ras* oncogene and transformation. J Virol 63:739–746, 1989.
6. Eliyahu D, Goldfinger N, Pinhasi-Kimhi O, Shaulsky G, Skurnik Y, Arai N, Rotter V, Oren M: Meth A fibrosarcoma cells express two transforming mutant p53 species. Oncogene 3:313–321, 1988.
7. Finlay CA, Hinds PW, Levine AJ: The p53 proto-oncogene can act as a suppressor of transformation. Cell 57:1083–1093, 1989.
8. Eliyahu D, Michalovitz D, Eliyahu S, Pinhasi-Kimhi O, Oren M: Wild-type p53 can inhibit oncogene-mediated focus formation. Proc Natl Acad Sci USA 86:8763–8767, 1989.
9. Baker SJ, Markowitz S, Fearon ER, Willson JKU, Vogelstein B: Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 249:912–915, 1990.
10. Diller L, Kassel J, Nelson CE, Gryka MA, Litwak G, Gebhardt M, Bressac B, Ozturk M, Baker SJ, Vogelstein B, Friend SH: p53 functions as a cell cycle control protein in osteosarcomas. Mol Cell Biol 10:5772–5781, 1990.
11. Johnson P, Gray D, Mowat M, Benchimol S: Expression of wild-type p53 is not compatible with continued growth of p53-negative tumor cells. Mol Cell Biol 11:1–11, 1991.
12. Mercer WE, Shields MT, Amin M, Sauve GJ, Appella E, Romano JW, Ullrich SJ: Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. Proc Natl Acad Sci USA 87:6166–6170, 1990.
13. Chen P-L, Chen Y, Bookstein R, Lee W-H: Genetic mechanisms of tumor suppression by the human p53 gene. Science 250:1576–1579, 1990.
14. Levine AJ, Momand J: Tumor suppressor genes: The p53 and retinoblastoma sensitivity genes and gene products. Biochim Biophys Acta 1032:119–136, 1990.
15. Lane DP, Benchimol S: p53: Oncogene or anti-oncogene? Genes Devel 4:1–8, 1990.
16. Crook T, Wrede D, Tidy J, Scholefield J, Crawford L, Vousden KH: Status of c-myc, p53 and retinoblastoma genes in human papillomavirus positive and negative squamous cell carcinomas of the anus. Oncogene 6:1251–1257, 1991.
17. Sidransky D, von Eschenbach A, Tsai YC, Jones P, Summerhayes I, Marshall F, Paul M, Green P, Hamilton SR, Frost P, Vogelstein B: Identification of p53 gene mutations in bladder cancers and urine samples. Science 252:706–708, 1991.
18. Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P, Glover T, Collins FS, Weston A, Modali R, Harris CC, Vogelstein B: Mutations in p53 gene occur in diverse human tumour types. Nature 342:705–708, 1989.
19. Prosser J, Thompson AM, Cranston G, Evans HJ: Evidence that p53 behaves as a tumor suppressor gene in sporadic breast tumors. Oncogene 5:1573–1579, 1990.
20. Davidoff AM, Humphrey PA, Iglehart JK, Marks JR: Genetic basis for p53 overexpression in human breast cancer. Proc Natl Acad Sci USA 88:5006–5010, 1991.
21. Varley JM, Brammar WJ, Lane DP, Swallow JE, Dolan C, Walker RA: Loss of chromosome 17p13 sequences and mutation of p53 in human breast carcinomas. Oncogene 6:413–421, 1991.
22. Baker SJ, Preisinger AC, Jessup JM, Paraskeva C, Markowitz S, Willson JK, Hamilton S, Vogelstein B: p53 gene mutations occur in combination with 17p allelic deletions or late events in colorectal tumorigenesis. Cancer Res 50:7717–7722, 1990.
23. Hollstein MC, Peri L, Mandard AM, Welsh JA, Montesano R, Metcalf RA, Bak M, Harris CC: Genetic analysis of human esophageal tumors from two high incidence geographic areas: Frequent p53 base subsitutions and absence of *ras* mutations. Cancer Res 51:4102–4106, 1991.

24. Hollstein MC, Metcalf RA, Welsh JA, Montesano R, Harris CC: Frequent mutation of the p53 gene in human esophageal cancer. Proc Natl Acad Sci USA 87:9958–9961, 1990.
25. Tamura G, Kihana T, Nomura K, Terada M, Sugimura T, Hirohashi S: Detection of frequent p53 gene mutations in primary gastric cancer by cell sorting and polymerase chain reaction single-strand conformation polymorphism analysis. Cancer Res 51:3056–3058, 1991.
26. Malkin D, Li FP, Strong LC, Fraumeni JF, Jr., Nelson CE, Kim D, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA, Friend SH: Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science 250:1233–1238, 1990.
27. Bressac B, Kew M, Wands J, Ozturk M: Selective G to T mutations of p53 gene in hepatocellular carcinoma from Southern Africa. Nature 350:429–431, 1991.
28. Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC: Mutational hotspot in the p53 gene in human hepatocellular carcinoma. Nature 350:427–428, 1991.
29. Chiba I, Takahashi T, Nau MM, D'Amico D, Curiel DT, Mitsudomi T, Buchhagen DL, Carbone D, Piantadosi S, Koga H, Reissman PT, Slamon DJ, Holmes EC, Minna, JD: Mutations in the p53 gene are frequent in primary resected non-small cell lung cancer. Oncogene 5:1603–1610, 1990.
30. Iggo R, Gatter K, Bartek J, Lane D, Harris AL: Increased expression of mutant forms of p53 oncogene in primary lung cancer. Lancet 335:675–679, 1990.
31. Hensel CH, Xiang RH, Sakaguchi AY, Naylor AL: Use of the single strand conformation polymorphism technique and PCR to detect p53 gene mutations in small cell lung cancer. Oncogene 6:1067–1071, 1991.
32. Gaidano G, Ballerini P, Gong JZ, Inghirami G, Neri A, Newcomb EW, Magrath IT, Knowles DM, Dalla-Favera R: p53 mutations in human lymphoid malignancies: Association with Burkitt lymphoma and chronic lymphocytic leukemia. Proc Natl Acad Sci USA 88:5413–5417, 1991.
33. Ahuja H, Bar-Eli M, Advani SH, Benchimol S, Cline MJ: Alterations in the p53 gene and the clonal evolution of the blast crisis of chronic myelocytic leukemia. Proc Natl Acad Sci USA 86:6783–6787, 1989.
34. Menon AC, Anderson KM, Riccardi VM, Chung RY, Whaley JM, Yandell DW, Farmer GE, Freiman RN, Lee JK, Li FP, Barker DF, Ledbetter DH, Kleider A, Martuza RL, Gusella JF, Seizinger BR: Chromosome 17p deletions and p53 gene mutations associated with the formation of malignant neurofibrosarcomas in von Recklinghausen neurofibromatosis. Proc Natl Acad Sci USA 87:5435–5439, 1990.
35. Miller CW, Aslo A, Tsay C, Slamon D, Ishizaki K, Toguchida K, Yamamuro T, Lampkin B, Koeffler HP: Frequency and structure of p53 rearrangements in human osteosarcoma. Cancer Res 50:7950–7954, 1990.
36. Mulligan LM, Matlashewski GJ, Scrable HJ, Cavenee WK: Mechanisms of p53 loss in human sarcoma. Proc Natl Acad Sci USA 87:5863–5867, 1990.
37. Marks JR, Davidoff AM, Kerns BJ, Humphrey PA, Pence JC, Dodge RK, Clarke-Pearson DL, Iglehart JD, Bast RC, Jr., Berchuck A: Overexpression and mutation of p53 in epithelial ovarian cancer. Caner Res 51:2979–2984, 1991.
38. Isaacs WB, Carter BS, Ewing CM: Wild-type p53 suppresses growth of human prostate cancer cells containing mutant p53 alleles. Cancer Res 51:4716–4720, 1991.
39. Stratton MR, Moss S, Warren W, Patterson J, Clark J, Fisher C, Fletcher CD, Ball A, Thomas M, Gusterson BA, Cooper CS: Mutation of the p53 gene in human soft tissue sarcomas: Association with abnormalities of the RB1 gene. Oncogene 5:1297–1301, 1990.
40. Metzger AK, Sheffield VC, Duyk G, Daneshvar L, Edwards MS, Cogen PH: Identification of a germ-line mutation in the p53 gene in a patient with an intracranial ependymona. Proc Natl Acad Sci USA 88:7825–7829, 1991.
41. Dippold WG, Jay G, DeLeo AB, Khoury G, Old LJ: p53 transformation-related protein: Detection by monoclonal antibody in mouse and human cells. Proc Natl Acad Sci USA 78:1695–1699, 1981.
42. Benchimol S, Pim D, Crawford L: Radioimmunoassay of the cellular protein p53 in mouse and human cell lines. EMBO J 1:1055–1062, 1982.

43. Thomas R, Kaplan L, Reich N, Lane DP, Levine AJ: Characterization of human p53 antigen employing primate specific monoclonal antibodies. Virology 131:502–517, 1983.
44. Rogel A, Popliker M, Webb CG, Oren M: p53 cellular tumor antigen: Analysis of mRNA levels in normal adult tissues, embryos, and tumors. Mol Cell Biol 5:2851–2855, 1985.
45. Oren M, Maltzman W, Levine AJ: Post-translational regulation of the 54K cellular tumor antigen in normal and transformed cells. Mol Cell Biol 1:101–110, 1981.
46. Reich NC, Oren M, Levine AJ: Two distinct mechanisms regulate the levels of a cellular tumor antigen, p53. Mol Cell Biol 3:2143–2150, 1983.
47. Lane DP, Crawford LV: T antigen is bound to a host protein in SV40-transformed cells. Nature 278:261–263, 1979.
48. Linzer DIH, Levine AJ: Characterization of a 54K dalton cellular SV40 tumor antigen in SV40 transformed cells. Cell 17:43–52, 1979.
49. Sarnow P, Ho YS, William J, Levine AJ: Adenovirus E1B-58Kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54Kd cellular protein in transformed cells. Cell 28:387–394, 1982.
50. Mercer WE, Nelson D, DeLeo AB, Old LJ, Baserga R: Microinjection of monoclonal antibody to protein p53 inhibits serum-induced DNA synthesis in 3T3 cells. Proc Natl Acad Sci USA 79:6309–6312, 1982.
51. Mercer WE, Avignolo C, Baserga R: Role of the p53 protein in cell proliferation as studied by microinjection of monoclonal antibodies. Mol Cell Biol 4:276–281, 1984.
52. Crawford LV, Pim DC, Gurney EG, Goodfellow P, Taylor-Papadimitriou J: Detection of a common feature in several human tumor cell lines — a 53,000-dalton protein. Proc Natl Acad Sci USA 78:41–45, 1981.
53. Rotter V, Boss MA, Baltimore D: Increased concentration of an apparently identical cellular protein in cells transformed by either Abelson murine leukemia virus or other transforming agents. J Virol 38:336–346, 1981.
54. Rovinski B, Benchimol S: Immortalization of rat embryo fibroblasts by the cellular p53 oncogene. Oncogene 2:445–452, 1988.
55. Levine AJ, Finlay CA, Hinds PW: The p53 proto-oncogene and its product. In: Villarreal LP (ed): Common Mechanisms of Transformation by Small DNA Tumor Viruses. ASM Publications, Washington DC, 1989, pp 21–37.
56. Michalovitz D, Eliyahu D, Oren M: Overproduction of protein p53 contributes to simian virus 40-mediated transformation. Mol. Cell. Biol 6:3531–3536, 1986.
57. Eliyahu D, Michalovitz D, Oren M: Overproduction of p53 antigen makes established cells highly tumorigenic. Nature 316:158–160, 1985.
58. Pohl J, Goldfinger N, Radler-Pohl A, Rotter V, Schirrmacher V: p53 increases experimental metastatic capacity of murine carcinoma cells. Mol Cell Biol 8:2078–2081, 1988.
59. Wolf D, Harris N, Rotter V: Reconstitution of p53 expression in a nonproducer Ab-MuLV-transformed cell line by transfection of a functional p53 gene. Cell 38:119–126, 1984.
60. Shaulsky G, Goldfinger N, Ben-Ze'ev A, Rotter V: Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigenesis. Mol Cell Biol 10:6565–6577, 1990.
61. Hinds PW, Finlay CA, Frey AB, Levine AJ: Immunological evidence for the association of p53 with a heat shock protein, hsc70, in p53-plus-*ras*-transformed cell lines. Mol Cell Biol 7:2863–2869, 1987.
62. Herskowitz I: Functional inactivation of genes by dominant negative mutations. Nature 329:219–222, 1987.
63. Werness BA, Levine AJ, Howley PM: Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science 248:76–79, 1990.
64. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM: The E6 oncoprotein encoded by human papillomavirus 16 or 18 promotes the degradation of 53. Cell 63:1129–1136, 1990.
65. DeCaprio JA, Ludlow JW, Figge J, Shew J-Y, Huang C-M, Lee W-H, Marsilio E, Paucha

E, Livingston DM: SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell 54:275–283, 1988.
66. Whyte P, Buchkovich KJ, Horowitz JM, Friend SH, Raybuck M, Weinberg RA, Harlow E: Association between an oncogene and an anti-oncogene; the adenovirus Ela proteins bind to the retinoblastoma gene product. Nature 334:124–129, 1988.
67. Dyson N, Howley PM, Munger K, Harlow E: The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 243:934–937, 1989.
68. Mowat M, Cheng A, Kimura N, Bernstein A, Benchimol, S: Rearrangements of the cellular p53 gene in erythroleukaemia cells transformed by Friend virus. Nature 314:633–636, 1985.
69. Chow V, Ben-David Y, Bernstein A, Benchimol S, Mowat M: Multistage Friend erythroleukemia: Independent origin of tumor clones with normal or rearranged p53 cellular oncogenes. J Virol 61:2777–2781, 1987.
70. Rovinski B, Munroe D, Peacock J, Mowat M, Bernstein A, Benchimol S: Deletion of 5′-coding sequences of the cellular p53 gene in mouse erythroleukemia: A novel mechanism of oncogene regulation. Mol Cell Biol 7:847–853, 1987.
71. Ben-David Y, Prideaux VR, Chow V, Benchimol S, Bernstein A: Inactivation of the p53 oncogene by internal deletions and/or retroviral integration in erythroleukemic cell lines induced by Friend leukemia virus. Oncogene 3:179–185, 1988.
72. Munroe DG, Rovinski B, Bernstein A, Benchimol S: Loss of a highly conserved domain on p53 as a result of gene deletion during Friend-virus-induced erythroleukemia. Oncogene 2:621–624, 1988.
73. Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Preisinger AC, Jessup JM, vanTuinen P, Ledbetter DH, Barker DF, Nakamura Y, White R, Vogelstein B: Chromosome 17 deletions and p53 gene mutations in colorectal carcinoma. Science 244:217–221, 1989.
74. Hinds PW, Finlay CA, Quartin RS, Baker SJ, Fearon ER, Vogelstein B, Levine AJ: Mutant p53 cDNAs from human colorectal carcinomas can cooperate with *ras* in transformation of primary rat cells. Cell Growth Differ 1:571–580, 1990.
75. Michalovitz D, Halevy O, Oren M: Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. Cell 62:671–680, 1990.
76. Yewdell J, Gannon JV, Lane DP: Monoclonal antibody analysis of p53 expression in normal and transformed cells. J Virol 59:444–452, 1986.
77. Gannon JV, Greaves R, Iggo R, Lane DP: Activating mutations in p53 produce a common conformational effect: A monoclonal antibody specific for the mutant form. EMBO J 9:1595–1602, 1990.
78. Martinez J, Georgoff I, Martinez J, Levine AJ: Cellular localization and cell cycle regulation by a temperature sensitive p53 protein. Genes Dev 5:151–159, 1991.
79. Gannon JV, Lane DP: Protein synthesis required to anchor a mutant p53 protein which is temperature-sensitive for nuclear transport. Nature 349:802–806, 1991.
80. Ginsberg D, Michael-Michalovitz D, Ginsberg D, Oren M: Induction of growth arrest by a temperature-sensitive p53 mutant is correlated with increased nuclear localization and decreased stability of the protein. Mol Cell Biol 11:582–585, 1991.
81. Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M: Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature 352:345–347, 1991.
82. Masuda H, Miller C, Koeffler HP, Battifora H, Cline MJ: Rearrangement of the p53 gene in human osteogenic sarcomas. Proc Natl Acad Sci USA 84:7716–7719, 1987.
83. Benchimol S, Lamb P, Crawford LV, Sheer D, Shours TB, Bruns GAP, Peacock J: Transformation associated p53 protein is encoded by a gene on human chromosome 17. Somat Cell Mol Genet 11:505–509, 1985.
84. Isobe M, Emanuel BS, Givol D, Oren M, Croce CM: Localization of gene for human p53 tumor antigen to band 17p13. Nature 320:84–85, 1986.

85. McBride OW, Merry D, Givol D: The gene for human p53 cellular tumor antigen is located on chromosome 17 short arm (17p13). Proc Natl Acad Sci USA 83:130–134, 1986.
86. Takahashi T, Nau MM, Chiba I, Birrer MJ, Rosenberg RK, Vinocour M, Levitt M, Pass H, Gazdar AF, Minna JD: p53: A frequent target for genetic abnormalities in lung cancer. Science 246:491–494, 1989.
87. Hollstein M, Sidransky D, Vogelstein B, Harris CC: p53 mutations in human cancers. Science 253:49–53, 1991.
88. Lavigueur A, Maltby V, Mock D, Rossant J, Pawson T, Bernstein A: High incidence of lung, bone, and lymphoid tumors in transgenic mice overexpressing mutant alleles of the p53 oncogene. Mol Cell Biol 9:3982–3991, 1989.
89. Li FP: Cancer families: Human models of susceptibility to neoplasia — The Richard & Hinda Rosenthal Foundation Award Lecture. Cancer Res 48:5381–5386, 1988.
90. Li FP, Fraumeni JF, Mulvihill JJ, Blattner WA, Dreyfus MG, Tucker MA, Miller RM: A cancer family syndrome in twenty-four kindreds. Cancer Res 48:5358–5362, 1988.
91. Srivastava S, Zou Z, Pirollo K, Blattner W, Chang EH: Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature 348:747–749, 1990.
92. Shaulsky G, Goldfinger N, Rotter V: Alterations in tumor development in vivo mediated by expression of wild-type or mutant p53 proteins. Cancer Res 51:5232–5237, 1991.
93. Bargonetti J, Friedman PN, Kern SE, Vogelstein B, Prives C: Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. Cell 65:1083–1091, 1991.
94. Tan T-H, Wallis J, Levine AJ: Identification of the p53 protein domain involved in formation of the simian virus 40 large T antigen-p53 protein complex. J Virol 59:574–583, 1986.
95. Raycroft L, Schmidt JR, Yoas K, Lozano G: p53 growth suppression correlates with transcriptional activation. Mol Cell Biol, in press, 1991.
96. Fields S, Jang SK: Presence of a potent transcription activating sequence in the p53 protein. Science 249:1046–1049, 1990.
97. Milner J, Medcalf EA: Cotranslation of activated mutant p53 with wild-type drives the wild-type p53 protein into the mutant conformation. Cell 65:765–774, 1991.
98. Halevy O, Michalovitz D, Oren M: Different tumor-derived p53 mutants exhibit distinct biological activities. Science 250:113–116, 1990.
99. Deppert W, Buschhausen-Denker G, Patschinsky T, Steinmeyer K: Cell cycle control of p53 in normal (3T3) and chemically transformed (MethA) mouse cells. II. Requirement for cell cycle progression. Oncogene 5:1701–1706, 1990.
100. Shohat O, Greenberg M, Reisman D, Oren M, Rotter V: Inhibition of cell growth mediated by plasmids encoding p53 anti-sense. Oncogene 1:277–283, 1987.
101. Soussi T, Caron de Fromental C, May P: Structural aspects of the p53 protein in relation to gene evolution. Oncogene 5:945–952, 1990.
102. Addison C, Jenkins JR, Sturzbecher HW: The p53 nuclear localisation signal is structurally linked to a $p34^{cdc2}$ kinase motif. Oncogene 5:423–426, 1990.
103. Dang CV, Lee WMF: Nuclear and nucleolar targeting sequences of *c-erb*, *c-myc*, *N-myc*, p53, HSP70 and HIV *tat* proteins. J Biol Chem 264:18019–18023, 1989.
104. Milner J, Medcalf EA, Cook AC: Tumor suppressor p53: Analysis of wild-type and mutant p53 complexes. Mol Cell Biol 11:12–19, 1991.
105. Lane DP, Gannon J: Cellular proteins involved in SV40 transformation. Cell Biol Int Rep 7:513–514, 1983.
106. Steinmeyer K, Deppert W: DNA binding properties of murine p53. Oncogene 3:501–507, 1988.
107. Kern SE, Kinzler KW, Baker SJ, Nigro JM, Rotter V, Levine AJ, Friedman P, Prives C, Vogelstein B: Mutant p53 proteins bind DNA abnormally in vitro. Oncogene 6:131–136, 1991.

108. Kern SE, Kinzler KW, Bruskin A, Jarosz D, Friedman P, Prives C, Vogelstein B: Identification of p53 as a sequence-specific DNA-binding protein. Science 252:1708–1711, 1991.
109. Raycroft L, Wu H, Lozano G: Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. Science 249:1049–1051, 1990.
110. O'Rourke RW, Miller CW, Kato GJ, Simon KJ, Chen D-L, Dang CV, Koeffler HP: A potential transcriptional activation element in the p53 protein. Oncogene 5:1829–1832, 1990.
111. Weintraub H, Hauschka S, Tapscott SJ: The MCK enhancer contains a p53 responsive element. Proc Natl Acad Sci USA 88:4570–4571, 1991.
112. Santhanam U, Ray A, Sehgal PB: Repression of the interleukin 6 gene promoter by p53 and the retinoblastoma susceptibility gene product. Proc Natl Acad Sci USA 88:7605–7609, 1991.
113. Gannon JV, Lane DP: p53 and DNA polymerase α compete for binding to SV40 T antigen. Nature 329:456–458, 1987.
114. Braithwaite AW, Sturzbecher H-W, Addison C, Palmer C, Rudge K, Jenkins JR: Mouse p53 inhibits SV40 origin-dependent DNA replication. Nature 329:458–460, 1987.
115. Sturzbecher HW, Brain R, Maimets T, Addison C, Ridge K, Jenkins JR: Mouse p53 blocks SV40 DNA replication in vitro and downregulates T antigen DNA helicase activity. Oncogene 3:405–413, 1988.
116. Wang EH, Friedman PN, Prives C: The murine p53 protein blocks replication of SV40 DNA in vitro by inhibiting the initiation functions of SV40 large T-antigen. Cell 57:379–392, 1989.
117. Friedman PN, Kern SE, Vogelstein B, Prives C: Wild-type, but not mutant, human p53 proteins inhibit the replication activities of simian virus 40 large tumor antigen. Proc Natl Acad Sci USA 87:9275–9279, 1990.

18. Nucleoside diphosphate kinases, *nm23*, and tumor metastasis: Possible biochemical mechanisms

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Introduction: Genetic control of tumor metastasis

Tumor metastasis remains the leading cause of death for cancer patients, excluding those with skin cancers. Metastasis is a complex process, requiring tumor cell invasion, motility, arrest and extravasation from the circulatory system, angiogenesis, and avoidance of host immune responses. A considerable list of proteases, motility factors, and adhesion molecules for cells and extracellular matrix components have been examined for their potential roles in the tumor metastatic process [reviewed in 1,2].

Less clear in the research literature are the molecular mechanisms, if any, that induce or coordinate the various phenotypic changes in metastasis. Two general regulatory themes for the metastatic process have been explored to date. First, at least 10 different oncogenes have been demonstrated in transfection experiments to induce or augment the tumor metastatic phenotype, as well as inducing tumorigenesis [reviewed in 3]. Acquisition of metastatic behavior by these oncogene-transfected cell lines was accompanied in many cases by alterations in the expression of proteases and adhesion molecules [3]. This observation is consistent with the hypothesis that oncogenes may directly or indirectly regulate the expression of a cascade of metastasis-related genes. It is important to note, however, that many of these oncogenes induced metastatic behavior in some but not all cell types tested. Factors such as cell type, state of differentiation, and expression of genes that modify oncogene action (i.e., *ras* and GAP, *k-rev*, exchange factors) may also be critical.

A second theme in the control of metastasis proposes that it may represent a reversion to a more embryonic state. Genes controlling differentiation may negatively regulate metastasis by preventing this reversion [reviewed in 2,4]. Studies with butyrate induction of differentiation are consistent with this hypothesis [5]. Hart [4] suggested that the two theories may merge, in that oncogenes may induce both cell division and phenotypes characteristic of embryonic cells. Support for this hypothesis is found in recent investigations of the roles of oncogenes, i.e., certain growth factors and growth factor

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receptors, in mediating characteristics such as cell motility in embryonic development [reviewed in 6].

Structure and expression of *nm23* in tumor metastasis

The *nm23* gene was identified by differential colony hybridization of low metastatic potential and high metastatic potential murine K-1735 melanoma cell lines [7]. Steady-state *nm23* RNA levels were quantitatively higher in two low metastatic potential K-1735 melanoma cell lines than in five related, high metastatic potential K-1735 melanoma cell lines. Quantitative reductions in *nm23* steady-state RNA levels were subsequently observed in high metastatic potential tumor cells in three additional rodent metastasis model systems, N-nitrososmethylurea (NMU) induced rat mammary tumors [7], c-Ha-*ras* + Ad2 *E1a* transfectd rat embryo fibroblasts [8], and mouse mammary tumor virus (MMTV) induced mouse mammary tumors [9].

In the study of oncogene regulation of cancer progression, a significant number of cases exist in which data observed in rodent model systems was not applicable to human tumor progression. Examples include the role of *ras* and certain *int* genes in human breast cancer, as predicted by the NMU and MMTV rodent model systems, respectively [reviewed in 10]. Therefore, *nm23* structure and expression has been investigated in four human cancer cell types. Two human *nm23* genes have been identified, *nm23-H1* [11] and *nm23-H2* [12], both of which encode 17 kDa proteins approximately 90% identical to the *nm23-1* murine protein and to each other. At the present time it is not known whether additional members of the *nm23* family of genes exist. Data concerning *nm23-H1* and *nm23-H2* structure and expression in human carcinomas of the breast, lung, colorectal tract, as well as childhood neuroblastoma, have provided an important insight: Many types of alterations to *nm23*, not just its reduced expression, occur in nature and are associated with high tumor metastatic potential.

The most comprehensive analysis of *nm23* structure and expression has been published in human breast cancer. We initially determined steady-state *nm23* RNA levels in tumor cells of sections of 17 human infiltrating ductal carcinomas using in situ hybridization [13]. All patients with evidence of lymph node metastases at surgery (considered the best indicator of high metastatic potential) had how steady-state *nm23* RNA levels. Patients with lymph node-negative (no evidence of metastasis) tumors had varied, but generally higher *nm23* RNA levels, consistent with data from the rodent metastasis systems. Studies by Hennessy et al. [14] confirmed and extended these observations. Total cellular RNA was extracted from 70 breast carcinomas and was analyzed for *nm23* steady-state RNA levels on Northern blots. The data were then compared to patient clinical course. Low tumor steady-state RNA levels were significantly associated with reduced patient

overall and disease-free survival. This association applied to the entire cohort as well as the lymph-node-negative and well-differentiated subsets.

The data indicate the potential utility of *nm23* expression as a prognostic indicator in breast cancer. Since tests at the RNA level require relatively large amounts of tumor material and techniques not consistently available in hospital histopathology laboratories, more recent studies have focused on immunoperoxidase staining of nm23 protein levels. Two small-scale studies have been reported to date. We prepared an affinity-purified polyclonal antibody to a synthetic nm23 peptide (anti-nm23 peptide 11 antibody) [11], which recognizes 17- and 18.5-kDa bands from lysates of human tumor cells on Western blots. In experiments not published, bacterial recombinant nm23-H1 protein migrated on SDS-PAGE with an apparent molecular weight of 18.5 kDa, while recombinant nm23-H2 protein migrated at 17 kDa. Thus anti-nm23 peptide 11 antibody recognized both gene products. Immunoperoxidase staining of 39 infiltrating ductal carcinomas was evaluated independently by three pathologists who were blind to clinical course data [15]. Patients with uniform or focal low-staining tumors exhibited a significantly reduced overall survival than those with uniformly high-staining tumors when analyzed by two of three pathologists. The clinical outcome correlation by the third pathologist closely approached significance ($p = 0.052$). Like the survival data, Cox's regression analysis indicated that nm23 staining was a significant independent predictor of reduced patient survival in two of the three pathologists' analysis and closely approached significance in the third ($p = 0.066$). A second study by Hirayama et al. [16] used a polyclonal antibody to a rat homolog of nm23 protein. Twenty-four breast carcinomas, consisting of intraductal carcinomas (two cases), invasive carcinomas (15 cases), invasive carcinomas with a predominant intraductal component (five cases) and mucinous carcinomas (two cases) were stained for NDPK protein, and the data were compared to patient relapse over a 5-year follow-up period. None of the patients with high staining tumors relapsed over the follow-up period, while 4 of 11 patients with intermediate staining tumors relapsed and 4 of 7 patients with low NDPK staining tumors relapsed over the same period. Taken together, the data suggest that traditional immunohistochemical staining should be evaluated for its prognostic potential in breast cancer, focusing on subgroups of patients in which current techniques are not sufficient, such as lymph-node-negative patients. The data are too limited at this point to definitively determined the prognostic potential of nm23 protein expression in human breast cancer.

The mechanism(s) by which nm23 expression is reduced in breast cancer are under study. We have reported that 64% of informative (heterozygous) breast tumors in a French cohort exhibited somatic allelic deletion of *nm23-H1* [17]. The *nm23-H1* gene was localized to chromosome 17q21 in the C.E.P.H. database [17]. The effect of allelic deletion at *nm23-H1*, potential allelic deletion of *nm23-H2*, and/or mutation of either gene, is currently under study. Additional investigations of the effects of hormones relevant to

breast development and carcinogenesis, as well as breast cancer oncogenes such as *c-erb-B-2*, are also under investigation.

Similar trends in *nm23* structure and expression in human lung cancer have been observed. In a blinded cohort of non-small cell lung carcinomas, 42% of informative tumors exhibited allelic deletion of *nm23-H1* [17]. All of the tumors with *nm23-H1* allelic deletions were adenocarcinomas, while allelic deletions at or near the *p53* tumor suppressor locus (at chromosome 17p13) were most prevalent in the squamous cell carcinomas. Since allelic deletion data may pertain to the gene used as the probe on the Southern blots or to a closely linked gene, we have also investigated *nm23* expression in a human lung cancer malignant progression model system [18]. A cell line established from SV40 immortalized human bronchial epithelial cells (BEAS-2B) was transfected/infected with v-Ki-*ras*, N-*ras*, or v-Ha-*ras*, generating the transformed BVK, BZN, and BZR cell lines, respectively. An additional cell line was established from the subcutaneous tumors resulting from injection of the BVK, BZN, and BZR lines, designated BVK-T11, BZN-T33, and BZR-T46, respectively, and these latter lines exhibited increased tumorigenic and/or malignant behavior upon subsequent injection into nude mice. We observed that *nm23* steady-state RNA levels, as well as nm23 protein biosynthetic levels, were progressively reduced with increasing malignant potential in the BEAS-2B-BVK-BVK-T11 and BEAS-2B-BZN-BZN-T33 systems. No consistent trend in expression was observed in the Ha-*ras* system. The data indicate that Ki-*ras* and N-*ras* can result in decreased *nm23* expression, which is intriguing in light of the fact that Ki-*ras* is the most prevalent *ras* mutation in adenocarcinomas of the lung, in which *nm23-H1* allelic deletion was observed [18]. The data also indicate that reduced *nm23* expression was not always correlated with malignant progression, as in the case of Ha-*ras*, and suggests that either other types of *nm23* alterations or mechanisms independent of *nm23* are operative.

Evidence for alterations in *nm23* structure and expression, other than its reduced expression, was observed in human colorectal carcinoma. Haut et al. [19] initially reported that *nm23* steady-state RNA levels were elevated in colonic polyps and carcinomas, as compared to adjacent normal colonic epithelium. Furthermore, no consistent trend was observed between carcinomas of Dukes grades A–D (exhibiting increasing evidence of local and distant metastases). Somatic allelic deletion of *nm23-H1* was observed in 20% of informative colorectal carcinomas examined [17]. Based on this observation, Cohn et al. [20] investigated in a prospective study of colorectal carcinoma patients whether *nm23-H1* allelic deletion was associated with the development of distant metastases. Twenty-one patients, free of distant metastases at initial surgery and heterozygous at the *nm23-H1* locus, were typed for *nm23-H1* allelic deletion in the tumor tissue and were followed for an average of 22 months for the development of distant metastases. Approximately 73% of patients with *nm23-H1* allelic deletions developed distant metastases, while only 20% of patients whose tumor was nondeleted

at the *nm23-H1* locus developed distant metastases; the data indicated a significant difference in the incidence of distant metastasis development by the log rank test ($p = 0.03$). In the *nm23-H1* allelic deletion subset, three patients initially stage I at surgery were present. These patients have a traditionally favorable (>95% chance) of recovery, and it was interesting that 2 of the 3 patients developed distant metastases. The data indicate that *nm23-H1* allelic deletion, and not overall *nm23* RNA or protein levels, is associated with metastatic progression in colorectal carcinoma. Current investigations are examining the remaining alleles for possible mutations. It cannot be ruled out at this point that a gene closely linked to *nm23-H1*, and not *nm23-H1* itself, is the gene responsible for regulating metastasis development in colorectal carcinoma.

In childhood neuroblastoma another pattern of altered *nm23* regulation has been correlated with high tumor metastatic potential. Stage 3 and 4 neuroblastomas (highly aggressive tumors) were reported to exhibit elevated levels of a 19-kDa protein, for which partial amino acid sequence matched *nm23-H1* [21]. It was concluded that *nm23* overexpression in aggressive neuroblastomas was not due to gene amplification, and was linked to *myc* amplification. A second study performed in our laboratory has generated different conclusions [22]. When matched DNA samples from normal bone marrow and tumor tissue were examined on Southern blots, amplification of one *nm23-H1* and *nm23-H2* allele was observed in 4/4 tumors with *myc* amplification, as well as 0/10 stage 1 and 2, 2/7 stage 3, 3/6 stage 4, and 1/3 stage 4s tumors with one copy of *myc*. In addition, analysis of *nm23-H1* and *nm23-H2* cDNAs by the single strand conformational polymorphism (SSCP) technique indicated the presence of a minor population of cDNAs with altered conformations in addition to the wild-type bands. The altered SSCP conformers corresponded to the 5′ but not the 3′ halves of the cDNAs, suggestive of the presence of nm23 mutations. Amplification may therefore represent the tumor cell's response to a mutated allele. Clearly this cancer cell type has a complex pattern of altered I structure and expression, and current efforts are focusing on identifying specific bases that are mutated.

Transfection of *nm23*

The fact that altered structure and/or expression of *nm23* has been correlated to metastatic progression in diverse tumor cell types argues for a functional role for *nm23* in the metastatic process. However, in no case can the data exclude the possibility that a closely linked gene is the actual metastasis regulatory gene and that variations in *nm23* are a 'bystander' effect of the amplification or deletion of relatively large portions of tumor DNA. Transfection data are therefore the only accepted indicator of a functional role for a putative suppressor gene.

Our initial transfection experiments utilized the murine K-1735 melanoma

metastasis model system, from which the *nm23* gene was identified. The most stable, highly metastatic cell line, K-1735 TK, was cotransfected with pRSVneo and pnm23-1 (containing the murine *nm23-1* cDNA, which was linked to a constitutive SV40 early promoter). Clones exhibiting both a 0.8-kb endogenous *nm23* band and a 1.0-kb band of transfected *nm23-1* on Northern blots were selected for analysis [23]. As controls, TK cells were transfected with pRSVneo, or with both pRSVneo and *pnm23-1*, and clones were selected that did not express the exogenous 1.0-kb band on Northern blots.

The tumor metastatic potential of the *nm23-1* and control transfected TK cells was determined by injection into mice. In experimental metastasis assays tumor cells were injected into the lateral tail veins, and pulmonary metastases was quantitated several weeks postinjection. TK clones expressing the *nm23-1* construct produced 57–96% fewer pulmonary metastases than did control transfected TK clones [23]. In spontaneous metastasis assays, tumor cells were injected subcutaneously, and the development of both primary tumors and metastases were measured. TK clones transfected with *nm23-1* produced fewer primary tumors than control transfectants, although the sizes of the tumors that were produced did not consistently differ from those of control transfectants. The formation of metastases in spontaneous metastasis assays is slow and inefficient, but significantly fewer animals that received *nm23-1*-transfected TK clones developed spontaneous metastases than animals receiving control-transfected TK clones [23]. The data indicate that *nm23-1* is a suppressor gene for primary tumor incidence and metastasis in K-1735 TK melanoma cells.

The mechanism of *nm23-1* action was investigated [23]. The endogenous TK *nm23* gene was PCR amplified and cloned, and its sequence was determined to be wild type. Thus, the *nm23-1* transfection did not supplement a mutated TK *nm23* gene with a wild-type gene. *Nm23-1*-transfected TK clones exhibited an altered response to the cytokine transforming growth factor-B_1 (TGF-B) in soft agar colonization assays. Control, highly metastatic TK clones were stimulated by TGF-B in a dose-dependent manner, while the lower metastatic potential, *nm23-1*-transfected TK clones showed no significant response to TGF-B [23]. Thus, *nm23-1* may exert an inhibitory effect on primary tumor incidence and metastasis by abrogating a stimulatory colonization response to TGF-B.

Homologs of *nm23* are involved in development and differentiation

Homologs of *nm23* have been identified in the fruit fly (*Drosophila melanogaster*) and cellular slime molds (*Dictyostelium discoideum*). The *Drosophila abnormal wing discs* (*awd*) gene encodes a 17-kDa protein that is 77% identical and 96% conserved in charge and hydrophobicity to the amino acid sequence of nm23-H1 [11]. Mutation or reduced expression of

awd permitted nearly normal larval development. After metamorphosis, when presumptive adult tissue differentiates, widespread abnormalities were observed. These include altered cell morphology, aberrant differentiation and cell necrosis, and the wing, leg, eye-antennae imaginal discs, ovaries, brain, and proventriculus [24–26]. An equally striking characteristic of *awd* mutants is the heterogeneity in the appearance of these abnormalities, to the extent that two wing discs from the same animal were often dissimilar. Most importantly, *awd* mutations have been functionally linked to differentiation and development. Transformation of the mutant germline with wild-type *awd* DNA resulted in normal development [26].

The gip 17 cDNA clone was identified from an cDNA expression library from cellular slime mold, screened with an antibody to kinases [27,28]. The predicted amino acid sequence of *gip* 17 is 66% identical to nm23-H1. RNA levels corresponding to *gip17* were determined in the nutrient-starvation-induced model of *Dictyostelium* differentiation [28]. Cells in the vegetative, proliferative state expressed high steady-state *gip17* RNA levels. Upon starvation cells begin to migrate, and *gip17* RNA levels were reduced. Continued starvation resulted in the aggregation and differentiation of the cells, and was accompanied by progressively increased levels of *gip17* RNA.

These evolutionary associations suggest that *nm23* may participate in the development and differentiation of mammalian organisms. The role of *nm23* in tumor metastasis may therefore involve its regulation of one or more 'differentiated' characteristics, which serve to stabilize functions such as cell division, adhesion, invasion, or migration. Two correlations of high nm23 protein levels with advanced differentiation have been noted in mammalian cells: Rat PC12 pheochromocytoma cells, when cultured with nerve growth factor or dibutryl cAMP, cease dividing, and produce axons characteristic of the differentiated state. Under these more differentiated conditions, nm23 protein levels are increased (personal communication, Dr. David Berstein). Second, immunoperoxidase staining of nm23 in squamous cell carcinomas of the head and neck indicate that differentiated (keratinizing) tumor cells express higher nm23 protein than undifferentiated tumor cells (personal communication, Drs. Edward Barker and Paul Pavolich).

Nucleoside diphosphate kinases and *nm23*

Nucleoside diphosphate kinases (NDPK) catalyze the phosphorylation of nucleoside diphosphates (NDP) into nucleoside triphosphates (NTP). The enzyme may be more correctly termed a *transferase* than a *kinase*, as a terminal phosphate is shifted from one nucleotide diphosphate to another. The cellular NDPK is an oligomer of three to six monomeric units, depending upon the cell type. Post-translational modification of NDPK and binding to other proteins have also been postulated. The biological functions of NDPK are not well understood. Virtually any biochemical process that requires a

NTP can be a candidate role for NDPK, although GTP function in microtubule assembly and G-protein function have received widespread attention. In addition, NDPK have been proposed to maintain intracellular pools of NTPs and to be part of the DNA replicon. Two NDPKs were purified from sequences from human erythrocytes and were identical in amino acid sequence to nm23-H1 and nm23-H2 [29]. In addition, *Drosophila awd* and *Dictyostelium gip17* have been demonstrated to encode NDPK [27,30].

In *Drosophila*, an association of differentiation abnormalities with reduced NDPK activity has been reported by Biggs et al. [30]. Mutant *awd* larvae contained 98% less NDPK activity than their wild-type counterparts. The remaining 2% of the NDPK activity was postulated to result from another NDPK gene or was maternally derived. Biggs et al. noted that mutant *awd* larvae exhibited condensed chromosomes, suggestive of colchicine-treated cells. Furthermore, awd protein was found to immunolocalize with tubulin. The data suggest that one consequence of *awd* mutation is the lack of NDPK provision of GTP or transphosphorylation of microtubule-bound GDP into GTP for efficient microtubule polymerization, with possible effects on cell shape, motility, mitosis, and chromosomal stability.

We have examined the K-1735 TK melanoma *nm23-1* and control-transfected clones for total NDPK activity, using both a spectrophotometric-coupled enzyme assay [30] and a radiosotopic assay [31]. Whereas nm23 RNA levels were elevated severalfold over controls, TK clones expressing the transfected *nm23-1* construct contained, on average, only 10% more NDPK than control-transfected clones (Table 1). Similarly, when the low

Table 1. The phenotypic changes induced in TK melanoma cells by NM23-1 transfection were accompanied by only small increases in cellular NDPK activity

	NDPK activity (umoles/min/mg)	Percent change
Experiment #1;		
A2 (control)	5.75	
A3 (nm23-1$^+$)	6.52	+13.4%
Experiment #2		
A2 (control)	5.86	
A3 (nm23-1$^+$)	6.53	+11.4%
Experiment #3		
A2 (control)	6.40	
A3 (nm23-1$^+$)	7.11	+13.6%
Experiment #4		
2–4 (control)	6.40	
4–6 (nm23-1$^+$)	7.11	+11.1%
Experiment #5		
2–4 (control)	5.95	
4–6 (nm23-1$^+$)	7.33	+23.2%
Experiment #6		
2–4 (control)	6.50	
4–6 (nm23-1$^+$)	7.26	+11.7%

Table 2. Lysates of tumor cells do not contain an inhibitor of NDPK activity

	NDPK activity (change in absorbance/min)	
	Expt #1	Expt #2
0.033 pg recombinant NM23-H1 protein	0.02	0.047
2.95 μg TK cell lysate	0.101	0.111
0.033 pg recombinant NM23-H1 protein + 2.95 μg TK cell lysate	0.131 108% of expected additive value	0.154 98% of expected of additive value

and high metastatic potential K-1735 melanoma cell lines clone 19 and TK were analyzed, significant differences in nm23 RNA levels lead to only minor changes in total NDPK activity (data not shown). Addition of cell lysate to a known amount of NDPK resulted in additive amounts of NDPK activity (Table 2), indicating that the lack of a significant amount of NTP produced in these assays was not due to the presence of an inhibitor of the NDPK assay in cell lysates or the excessive presence of NTPases. Subcellular fractionation of one set of *nm23-1*-transfected and control-transfected TK clones is shown in Table 3. In no subcellular fraction were severalfold increases in NDPK activity found. Analysis of chromosome condensation and tubulin organization, the latter shown in Fig. 1, also indicated no significant differences in the *nm23-1*- and control-transfected TK clones. The data argue that if the NDPK activity of *nm23-1* is responsible for the observed phenotypic changes upon transfection into TK cells, then specific mechanisms must be involved. The data also indicate that gross changes in microtubule organization were not required for alterations in the metastatic phenotype.

What biochemical mechanisms could account for the specificity of action of NDPK? NDPK have been demonstrated to associate with membrane signal transducing systems such as hormone-sensitive adenylate cyclase [32,33] and has been postulated to function as GTP supply machinery, resulting in their activation. The GTP supply in this system in activated by hormone but is insensitive to cholera toxin, the latter of which acts in a receptor-independent mechanism [34]. Complex formation between heterotrimeric G-protein Gs and membrane-associated NDPK was demonstrated in a detergent-solubilized preparation [35]. Two possible mechanisms have been postulated: (1) NDPK increases the GTP concentration in the immediate vicinity of the G protein, leading to increased GDP for GTP exchange and G protein activation. (2) Alternately, NDPK directly transphosphorylates GDP bound to the G protein into GTP. The discrimination of these possibilities is complicated by the difficulty in measuring GDP dissociation rates from G proteins and awaits further rigid evidence [36,37].

The possible interaction between NDPK and small molecular weight G

Table 3. NDPK activity of fractionated 2–4 (control-transfected) and 4–6 (NM23-1-transfected) TK cells

		NDPK activity (nmoles/min/mg)		
Cell fraction	Cell line	2–4 (control)	4–6 (NM23-1$^+$)	Percent change (4–6/2–4)
Experiment 1				
Whole homogenate		2330	2700	+16%
Nuclei enriched (150 g ppt)		1110	904	−19%
Mitochondria enriched (15 kg ppt)		556	402	−28%
Microsome enriched (100 kg ppt)		399	347	−13%
Cytoplasm (100 kg sup)		3940	4580	+16%
Plasma membrane		161	109	−32%
Experiment 2				
Whole homogenate		2110	2280	+8%
Nuclei enriched (150 g ppt)		950	944	−1%
Mitochondria enriched (15 kg ppt)		457	409	−11%
Microsome enriched (100 kg ppt)		205	399	95%
Cytoplasm (100 kg sup)		5090	6200	+22%
Plasma membrane		166	168	+1%

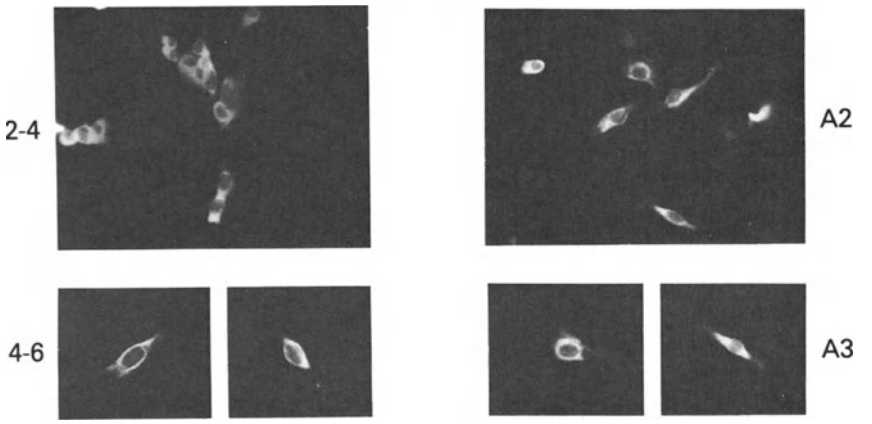

Figure 1. Immunostaining of tubulin in control and *nm23-1*-transfected K-1735 TK melanoma cells. Two sets of control and *nm23-1*-transfected TK clones were stained for tubulin expression, as previously described [30]. 2–4, control transfectant; 4–6, *nm23-1* expressing transfectant. A2, control transfectant; A3, *nm23-1* expressing transfectant.

proteins was suggested in a study of *Drosophila*. The x-linked *prune* (*pn*) eye color mutation has a specific lethal interaction with the *nm23* homolog *awd*. This lethal interaction occurs only upon mutation of an *awd* serine to a proline and is known as the *Killer of prune* mutation (*awd K-pn*) [24]. *Pn* was recently reported to demonstrate homology to the catalytic domain of mammalian GTPase activating protein (GAP) [38]. GAPs stimulate the GTPase activity of small G proteins such as *ras*. The data lead to the suggestion that nm23/awd/NDPK and a GAP-like protein may interact on the same signaling pathway as *ras*-like proteins [39]. Unpubished observations indicate no evidence for a direct association of *nm23* with *ras*, however. A 17-kDa protein was observed to copurify with *ras*, but it was unreactive to anti-nm23 peptide antibody (personal communication, Dr. C.W. Marshall).

Recombinant murine nm23-1 protein has been demonstrated to transphosphorylate GDP bound to a small G protein, ADP ribosylation factor (ARF), under conditions of undetectable GDP dissociation [40]. Activation of ARF was also quantitated, indicating that transphosphorylation had a functional effect [35]. Thus, NDPK may bind, transphosphorylate, and activate this small G protein. Two issues complicate an interpretation of the significance in vivo of these findings: First, the K_m and V_{max} were calculated for NDPK interaction with ARF-GDP as opposed to free GDP; ARF-GDP exhibited a lower K^m but a slower V_{max} than free GDP [35]. In vivo, ARF-GDP could represent (1) a stimulator of ARF activation, given the significant V_{max} and K_m, or (2) an inhibitor of ARF, by virtue of its tight binding to ARF and relatively slow catalysation of the phosphorylation reaction. Thus, the in vivo significance of NDPK interactions with small G proteins remains unclear.

If NDPK serve to activate small G proteins in vivo, the issue of specificity must also be addressed in terms of what small G proteins are responsive and what NDPK are capable. Does NDPK activate ARF, a subset of small G proteins, or virtually all G proteins?

It cannot be excluded that nm23 possess biochemical activities distinct from its NDPK activity, which mediate its biological effects. One candidate is a repeat of three leucines indicative of a leucine zipper, involved in protein-protein and protein-DNA interactions [12]. Site-directed mutagenesis underway in the laboratory will alter nm23-1 amino acids to eliminate these and other biochemical activities. The constructs will then be transfected into K-1735 TK cells to definitively link the biochemical activities with alterations in the metastatic phenotype.

Clinical perspectives

The phenotypic consequences of transfection of human *nm23* cDNAs into a variety of human tumor cell lines must be determined before any preclinical strategies for *nm23* can be developed. From a theoretical standpoint, if human *nm23* genes are suppressive for metastastic behavior, two strategies can be pursued. First, drugs that increase *nm23* expression can be identified and tested. However, if mutant nm23 proteins are found, as is suggested by the SSCP data in neuroblastoma, it is not known whether a simple increase in the synthesis of an wild-type gene will have a significant beneficial effect. Second, gene therapy offers the eventual hope for delivery of a wild-type construct to tumor cells, although significant advances in tissue-specific delivery, stability of the construct, and efficiency of infection are required.

References

1. Liotta LA, Steeg PS, Stetler-Stevenson WG: Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. Cell 64:327–336, 1991.
2. Nicolson GL: Tumor cell instability, diversification and progression to the metastatic phenotype: From oncooene to oncofetal expression. Cancer Res 47:1473–1487.
3. Steeg PS: Genetic control of the metastatic phenotype. Semin Cancer Biol 2:105–110, 1991.
4. Hart IR, Easty D: Tumor cell progression and differentiation in metastasis. Semin Cancer Biol 2:87–96, 1991.
5. Lotan R: Retinoids as modulators of tumor cell invasion and metastasis. Semin Cancer Biol 2:197–208, 1991.
6. Reid L: From gradients o axes, from morphogenesis to differentiation. Cell 63:759–767, 1991.
7. Steeg PS, Bevilaqua G, Kopper L, Thorgeirsson UP, Talmadge JE, Liotta LA, Sobel ME: Evidence for a novel gene associated with low tumor metastatic potential. J Natl Cancer Inst 80:202–204, 1988.
8. Steeg PS, Bevilacqua G, Pozzatti R, Liotta LA, Sobel ME: Altered expression of *nm23*, a

gene associated with low tumor metastatic potential, during adenovirus 2 *E1a* inhibition of experimental metastasis. Cancer Res 48:6550–6554, 1988.
9. Steeg PS, Bevilacqua G, Rosengard AM, Cioce V, Sobel ME, Liotta LA: Altered gene expression in tumor metastasis: The *nm23* gene. In: Schirrmacher V, Schwartz-Albiez R, (eds): Cancer Metastasis. Springer, Heidelberg, 1989, pp 48–52.
10. Van de Vijer MJ, Nusse R: The molecular biology of breast cancer. Biochem Biophy Acta 1072:33–50, 1991.
11. Rosengard AM, Krutzsch HC, Shearn A, Biggs JR, Barker E, Margulies IMK, King CR, Liotta LA, Steeg PS: Reduced nm23/awd protein in tumor metastasis and aberrant Drosophila development. Nature 342:177–180, 1989.
12. Stahl JA, Leone A, Rosengard AM, Porter L, King CR, Steeg PS: Identification of a second human *nm23gene*, *nm23-H2*. Cancer Res 51:445–449, 1991.
13. Bevilacqua G, Sobel ME, Liotta LA, Steeg PS: Association of low *nm23* RNA levels in human primary infiltrating ductal breast carcinomas with lymph node involvement and other histopathological indicators of high metastatic potential. Cancer Res 49:5185–5190, 1988.
14. Hennessy C, Henry JA, May FEB, Westley BR, Angus B, Lennard TWJ: Expression of the anti-metastatic gene *nm23* in human breast cancer; association with good prognosis. J Natl Cancer Inst 83:281–285, 1991.
15. Barnes R, Masood S, Barker E, Rosengard AM, Coggin DL, Crowell T, King CR, Porter-Jordan K, Wargotz E, Liotta LA, Steeg PS: Low nm23 protein expression in infiltrating ductal breast carcinoma correlates with reduced patient survival. Am J Pathol 139:245–250, 1991.
16. Hirayama R, Sawai S, Takagi Y, Mishima Y, Kimura N, Shimada N, Esaki Y, Kurasjima C, Hirokawa K: Positive relationship between expression of anti-metasatic factor (nm23 gene product or nucleoside diphsophate kinase) and good prognosis in human breast cancer. J Natl Cancer Inst 83:1249–1250, 1991.
17. Leone A, McBride OW, Weston A, Wang MG, Anglard P, Cropp CS, Goepel JR, Lidereau R, Callahn RC, Linehan WM, Rees R, Harris CC, Liotta LA, Steeg PS: Somatic allelic deletion of nm23-H1 in human cancer. Cancer Res 51:2490–2493, 1991.
18. Rosengard AM, Forrester K, Pfeifer A, Stahl JA, Der C, Reddel R, Harris CC, Steeg PS: Malignant progression of v-Ki-ras and N-ras, but not v-Ha-ras transformed bronchial epithelial cells correlates with reduced nm23 expression. Manuscript submitted for publication.
19. Haut M, Steeg PS, Willson JVK, Markowitz SD: Induction of nm23 gene expression in human colonic neoplams and equal expression in colon tumors of high and low metastatic potential. J Natl Cancer Inst 83:712–716, 1991.
20. Cohn KH, Wang F, De-Soto-LaPaix F, Solomon WB, Paterson LG, Arnold MR, Weimar J, Feldman JG, Levy A, Leone A, Steeg PS: Association of nm23-H1 allelic deletions with distant metastases in colorectal carcinoma. Lancet 338:722–724.
21. Hailat N, Keim DR, Melhem RF, Zhu X, Eckerskorn C, Brodeur GM, Reynolds CP, Seeger RC, Lottspeich F, Strahler JR, Hanash SM: High levels of p19/nm23 protein in neuroblastoma are associated with advance stage disease and with N-myc amplification. J Clin Invest 88:341–345, 1991.
22. Leone A, Seeger RC, Slamon DJ, Steeg PS: Amplification and altered conformation of nm23-H1 and nm23-H2 in childhood neuroblastoma. Oncogene, in press.
23. Leone A, Flatow U, King CR, Sandeen MA, Margulies IMK, Liotta LA, Steeg PS: Reduced tumor incidence, metastatic potential and cytokine responsiveness of nm23-transfected melanoma cells. Cell 65:25–35, 1991.
24. Biggs J, Hersperger E, Dearolf C, Shearn A: Analysis of the lethal interaction between the *prune* and *Killer of prune* mutations of Drosophila. Genes Dev 2:1333–1343, 1988.
25. Dearolf C, Hersperger E, Shearn A: Developmental consequences of awd^{b3}, a cell-autonomous lethal mutation of Drosophila induced by hybrid dysgenesis. Dev Biol 129:159–168, 1988.

26. Dearolf CR, Tripoulas N, Biggs J, Shearn A: Molecular consequences of *awd*^{b3}, a cell-autonomous lethal mutation of Drosophila induced by hybrid dysgenesis. Dev Biol 129:169–178, 1988.
27. Lacombe ML, Wallet V, Troll H, Veron M: Functional cloning of a nucleoside diphosphate kinase from *Dictyostelium discoideum*. J Biol Chem 265:10012–10018, 1990.
28. Wallet V, Mutzel R, Troll H, Barzu O, Wurster B, Veron M, Lacombe ML: Dictyostelium nucleoside diphosphate kinase highly homologous to nm23 and awd proteins involved in memmalian tumor metastasis and Drosophila development. J Natl Cancer Inst 82:1199–1202, 1990.
29. Gilles AM, Presecan E, Vonica A, Lascu I: Nucleoside diphosphate kinase from human erythrocytes. Structural characterization of the two polypeptide chains responsible for heterogeneity of the hexameric enzyme. J Biol Chem 266:8784–8789, 1991.
30. Biggs J, Hersperger E, Steeg PS, Liotta LA, Shearn A: A Drosophila gene which is homologous to a mammalian metastasis associated gene codes for a nucleoside diphosphate kinase. Cell 63:933–940, 1990.
31. Kimura N, Shimada N: GDP does not mediate but rather inhibits hormal signal to adenylate cyclase. J Biol Chem 258:2278–2283, 1983.
32. Kimura N, Shimada N: Membrane associated nucleoside diphosphate kinase from rat liver. J Biol Chem 263:4647–4653, 1988.
33. Otero AS: Transphospharylation and G protein activation. Biochem Pharmacol 39:1399–1404, 1990.
34. Kimura N, Shimada N: Differential susceptibility to GTP formed from added GDP via membrane-associated nucleoside diphosphate kinase of GTP-sensitive adenylate cyclase achieved by hormone and cholera toxin. Biochem Biophys Res Commun 131:199–206, 1985.
35. Kimura N, Shimada N: Evidence for complex formation between GTP binding protein (Gs) and membrane-associated nucleoside diphosphate kinase. Biochem Biophys Res Commun 168:99–106, 1990.
36. Kikkawa S, Takahashi K, Takanashi K, Shimada N, Ui M, Kimura N, Katada T: Conversion of GDP into GTP by nucleoside diphosphate kinase on the GTP-binding proteins. J Biol Chem 265:21536–21540, 1990.
37. Kikkawa S, Takahashi K, Takanashi K, Shimada N, Ui M, Kimura N, Katada T: Conversion of GDP into GTP by nucleoside diphosphate kinase on the GTP-binding proteins. J Biol Chem 266:12795, 1991.
38. Teng DH, Engele CM, Venkatesh TR: A product of the prune locus of Drosophila is similar to mammalian GTPase-activating protein. Nature 353:437–440, 1991.
39. Ruggieri R, McCormick F: Ras and the awd couple. Nature 353:390, 1991.
40. Randazzo P, Nortup JK, Kahn RA: Activation of a small GTP binding protein (ARF) by necleoside diphosphate kinase catalyzed phosphorylation of bound GDP. Science 254:850–853, 1991.

19. Angiogenesis: A mechanism by which oncogenes and tumor suppressor genes regulate tumorigenesis

Noël Bouck

Introduction

Normal cells become malignant in a tissue culture flask, in experimental animals, and in cancer patients, largely as a result of the accumulation of a series of genetic lesions that activate proto-oncogenes to oncogenes and that inactivate tumor suppressor genes, sometimes called anti-oncogenes [1]. About 50 oncogenes and a dozen anti-oncogenes have been cloned and sequenced. The functions of most of them are well understood in the sense that the primary structure and biochemical activity of the proteins they encode are known. The vast majority of these proteins are either transcription factors or participants in signal transduction pathways (see Chapter 1). However, the identification of an oncogene-encoded protein as a transcription factor or a member of a signal transduction pathway does not fully explain why an increase in its activity (or its loss of function in the case of the anti-oncogenic tumor suppressor genes) increases the likelihood that a cell will become tumorigenic. A complete understanding of how the activation of an oncogene contributes to tumorigenicity demands additional information. It requires (1) that the oncogene-regulated genes be identified and the function of their protein products determined and (2) that those oncogene-regulated proteins that play a crucial role in the development of the tumorigenic phenotype be differentiated from those that are irrelevant. One way to begin to identify crucial oncogene- and tumor suppressor gene-regulated proteins is to investigate molecules that control a phenotype known to be essential for tumor growth and then to determine if the production of such proteins is altered by oncogene activation or by suppressor gene loss. One phenotype that is particularly amenable to such analysis is angiogenesis — the ability to induce neovascularization or new blood vessel growth.

Induction and inhibition of neovascularization

In the normal adult organism in the absence of disease, there is no new blood vessel growth, except in response to injury or to cyclical changes in

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Table 1. Naturally occurring inducers and enhances of in vivo neovascularization

Growth factors	* Acidic fibroblast growth factor (aFGF)	[2,3][a]
	* Basic fibroblast growth factor (bFGF)	[2,3]
	* Epidermal growth factor (EGF)	[2,3][b]
	* Transforming growth factor alpha (TGF-α)	[2,3]
	* Transforming growth factor beta (TGF-β)	[2,3]
	* Tumor necrosis factor alpha (TNF-α)	[2,3]
	* Platelet-derived endothelial cell growth factor (PD-ECGF)	[2,3]
	* Vascular permability factor (VPF, VEGF, or FSdGF)	[2,3]
	* Interleukin 1 (IL-1)	[4–6][c]
	Interleukin 2 (IL-2)	[7]
Other proteins and peptides	* Angiogenin	[2,3]
	Angiotensin II	[9]
	* Plasminogen activator	[10]
	* Ceruloplasmin	[11]
	* Polyamines	[12]
	* Substance P	[13]
	Human angiogenic factor (h-AF)	[14,15]
	Fibrin	[3]
Carbohydrates	Hyaluronan fragments	[17,18]
	* Lactate	[19]
Lipids	* Prostaglandins E1, E2	[20]
	Monobutyrin	[3,20]
	12(R)-hydroxyeicosatrieonic acid (compound D)	[21]
Small molecules and ions	* Nicotinamide	[3]
	Adenosine diphosphate	[16,22–23]
	Selenium	[24]
	Copper	[16,25]
Others	* Endothelial cell stimulating angiogenesis factor (ESAF)	[26–28]
	Angiotropin	[2,3,29]

* Known to be produced by tumors and/or tumor cell lines.
[a] Reviews are cited for well-known inhibitors.
[b] See also contrary report [8].
[c] Inhibitory in some circumstances [36].

the female reproductive organs. Although many naturally occurring compounds are capable of inducing neovascularization (Table 1), in normal tissues they are apparently not available to the endothelial cell at effective concentrations. Some are awaiting activation, such as plasminogen activator, others are sequestered within cells such as the platelet-derived endothelial cell growth factor or within the interstitial matrix like basic fibroblast growth factor [47]. On the other hand, naturally occurring molecules capable of blocking angiogenesis (Table 2) are thought to be present at effective concentrations in normal tissues, where they may act to make the endothelial cell insensitive to modest ambient concentrations of inducers. Upon wounding, the stringent inhibition of angiogenesis immediately dissolves. In response to cytokines and enzymes released by cells involved in clotting and inflammation, new vessels grow to support the repair of injured tissue and, when healing is complete, regress to the preinjury level. Successful solid tumors usurp the inductive phase of this physiological wounding response,

Table 2. Naturally occurring inhibitors of in vivo neovascularization

* Platelet factor 4 (PF-4)	[see 2,3,31–34]
Eosinophil major basic protein	[see 3,31]
* protamine	[see 2,31,35]
Interleukin-1 (IL-1; bFGF specific?)	[36]
* Interferons	[see 2,37]
* Retinoids	[38–40]
* 1,25-dihydroxyvitamin D3	[30]
* Angiostatic steroids + sulphated polysaccharide	[see 2,3,41]
* Tissue inhibitor of metalloproteinases (TIMP)	[see 20,12,42]
* RNase inhibitor (angiogenenin specific)	[43]
* Laminin peptides	[44,45]
High mass hyaluronan	[17]
Thrombospondin (TSP)	[see 2,46]

* Shown to slow growth in vivo of some experimental tumors.

creating around them an environment akin to that of an active wound, complete with continuous neovascularization [48].

Mechanisms linking transformation and angiogenesis

A large body of work published over the last 20 years (principally from the Harvard laboratory of Judah Folkman) [reviewed in 20,49] indicates that solid tumors are absolutely dependent on this angiogenesis. Without it they are unable to grow progressively to a thickness of more than 1–2 mm or to metastasize efficiently [3,50,51]. All available evidence is consistent with this being just as true for human tumors as for those growing in experimental animals. For example, human melanomas become life threatening only when they expand to a thickness of greater than 0.75 mm, an event accompanied by the initiation of angiogenesis around the thickening tumor [52]. Cells in progressively growing solid tumors use a number of strategies to attract the vascular supply they need [reviewed in 2,3,53–57]: (1) They produce diffusible angiogenic factors that directly activate endothelial cells, stimulating them to sprout into vessels that grow towards the developing tumor. (2) They elaborate cytokines, which attract macrophages to their environment [58], mast cells [59], and neutrophils [60], which also produce angiogenic factors. (3) They halt the production of molecules that inhibit angiogenesis [20,61]. (4) They produce enzymes that release angiogenic factors stored in the extracellular matrix [62]. (5) They stimulate adjacent normal tissue to make enzymes such as stromelysin [63] and collagenase [64,65] that can be activated to promote angiogenesis.

If sequentially acquired lesions in oncogenes and tumor suppressor genes cause normal cells that are not angiogenic in situ to develop into neovascularizing tumors, it is a logical presumption that it is oncogene activation

and/or suppressor gene loss that directly or indirectly enables cells to become angiogenic. There are many instances in the current literature that can be interpreted as supporting a causative link between oncogenes and anti-oncogenes and angiogenesis, although this link has seldom been studied directly.

The angiogenic phenotype is acquired stochastically

It is during tumor initiation, promotion, and progression in vivo, as oncogenes are being activated and tumor suppressor genes lost, that the ability to be angiogenic arises. It can arise early, midway, or late in the tumorigenic process [49,66]. It is necessary, but of course not sufficient, for tumorigenicity, as is true of most individual lesions in oncogenes or anti-oncogenes.

Some oncogenes encode angiogenic factors

Constructs expressing EGF [67], TGF-α [68–70], or bFGF [71,72] can transform cells to increased tumorigenicity, and each of these genes encodes a growth factor that can induce angiogenesis. Three tumor-derived oncogenes — *int*-2, *hst*/K-*fgf*/KS3, and FGF-5 — are highly homologous to bFGF, the prototypic inducer of angiogenesis, and may encode angiogenic proteins, as they are more similar to bFGF than is angiogenic acidic FGF. The induction of angiogenesis is certainly not the only function of the secreted molecules encoded by these oncogenes, for they can be potent growth factors for the cells they transform in tissue culture systems, where angiogenesis is irrelevent. In most cases it is not yet clear whether the angiogenic molecules encoded by the transforming oncogenes are actually produced by the cells in sufficient quantity to directly induce all neovascularization necessary for progressive tumor growth in vivo. Most likely they have dual roles, activating endothelial cells directly and attracting accessory cells to amplify the response. A tight correlation has been found between bFGF release and angiogenesis in developing fibrosarcomas in transgenic mice carrying bovine papilloma virus [73]. Interestingly, neoplasms in these animals become aggressive and angiogenic not when the cells of the developing tumor begin to make large amounts of bFGF protein, but rather when these cells become able to export it efficiently. It is not yet clear whether the switch to a secretory mode is due to a change in the gene encoding bFGF, which in experimental systems would convert it to an oncogene [74], or to an alteration in cellular secretory pathways. Secretion begins at the same time that the developing tumor cells begin to overexpress the E6 and E7 oncoproteins encoded by the resident papilloma virus, raising the possibility that these viral oncogenes may be ultimately responsible for the initiation of angiogenesis in this system.

Some transforming oncogenes induce cells to produce angiogenic factors

Ras, *src*, and certain other oncogenes appear to enhance angiogenesis, for tumors grown from *ras*-transformed fibroblasts tend to be exceptionally bloody and to have more vessels than tumors derived from spontaneously transformed controls [75]. When cells from the mouse urogenital sinus are allowed to regenerate a prostatelike organ after transplantation under the kidney capsule, angiogenesis is increased by 10-fold if H-*ras*-expressing cells are present [76]. How *ras* stimulates angiogenesis is unknown but it can increase the expression of angiogenic TGF-α, as can other oncogenes [69]. H-*ras* expression can also raise phospholipase A2 activity in cultured rodent fibroblasts (as can *src*, *met*, *trk*, *mos*, and *raf*, but not *fos* or *myc*) [77], which could increase the production of angiogenic prostaglandins. The synthesis of prostaglandins was measured directly in chicken embryo fibroblasts (CEF) transformed by a variety of viral oncogenes, and PGE_2 was found to be increased by three- to fourfold in cells transformed by *src*, *fps*, *ros*, *yes*, *mos*, and *ras*, but not by v-*crk* or c-*src* [78]. Increased PGE production in v-*src*-transformed cells is proportional to the degree of transformation [77] and may be due to the oncogene-mediated persistent induction [79] and appropriate splicing [80] of the transcript encoding prostaglandin synthase. V-*src*-transformed CEF also acquire the ability at their cell surface to activate plasminogen activator [81], another molecule capable of inducing angiogenesis.

Oncogenes encoding transcription factors might be expected to influence the production of many molecules relevant to angiogenesis. For example, angiogenic molecules, such as collagenase and IL-2, are sensitive to transcriptional regulation via AP-1 promoter sites and thus represent potential targets genes for activated *fos* and *jun* [82]. As yet, however, no specific links between these oncogenes and angiogenesis have been made. A preliminary report from Schweigerer et al. [83] describes the ability of the N-*myc* oncogene to enhance the vascularization and malignant potential of neuroblastoma cells having low N-*myc* expression, apparently by increasing the production of endothelial cell growth factors. Enhanced vascularization and the increased metastasis that it engenders may contribute to the well-documented increased aggressiveness of neuroblastomas carrying amplified N-*myc* [84].

The tumorigenicity of DNA tumor viruses depends on the induction of angiogenesis

The viral oncogenes of DNA tumor viruses are probably able to make cells angiogenic, especially those oncogenes sufficient to convert nonangiogenic primary cultures into cells capable of forming well-vascularized solid tumors. In the case of middle T antigen of polyoma virus, we have some insight into a possible mechanism. Expression of this oncogene in endothelial cells of

transgenic or newborn mice results in high fibrinolytic activity due to the unusual combination of increased production of urokinase plasminogen activator (uPA) and decreased production of plasminogen activator inhibitors (PAI) [85]. When endothelial cells expressing middle T are injected into normal mice, hemangiomas grow rapidly but consist largely of host endothelial cells whose migration has been stimulated and whose differentiation has been inhibited apparently as a result of rampant matrix degradation triggered by the injected middle T-expressing cells [86]. UPA alone can be angiogenic (Table 1), and one can envision in another cell or another environment that polyoma middle T could induce angiogenesis via modulation of uPA and PAI to a more modest degree than occurs in the embryonic endothelial cell.

The large T antigen of SV-40 virus is capable of transforming cultured cells to tumorigenicity but is apparently incapable of providing an angiogenic phenotype when it is expressed in the beta cells of the pancreas in a transgenic mouse [87]. It is not clear if this is due to constraints on its level of expression imposed by the transgenic system or whether this simply indicates that the mechanisms by which a single oncogene induces angiogenesis are not effective in all cells and/or in all tissue environments. Many other viral oncogenes sufficient to transform cells in culture to tumorigenicity fail to cause every cell in a transgenic mouse in which they are expressed to produce tumors [88]. In two such cases, baby chicks infected with v-*src* expressing Rous sarcoma virus [89] and transgenic mice expressing v-*jun* driven by the H-2K promoter [90], tumors form only at the site of a wound, which is of course accompanied by angiogenesis. But the observation that tail fibroblasts cultured from v-*jun* transgenic mice are able to form vascular solid tumors in nude mice suggests that it is the further growth of the oncogene-containing cells, stimulated by the wounding environment in vivo, or by the analogous serum-containing culture medium in vitro, that is crucial in these models and not the angiogenesis.

The loss of tumor suppressor genes can activate angiogenesis

In two histologically different hamster tumor systems, loss of a suppressor gene influences angiogenesis. In a cultured BHK fibroblast line that is converted to anchorage independence and tumorigenicity by loss of a tumor suppressor gene, suppressor loss is invariably accompanied by a gain in the ability to be angiogenic [61]. In this system, the presence of an active tumor suppressor gene ensures that the normal cell will produce high levels of mRNA encoding a secreted inhibitor of angiogenesis, thrombospondin [46, 61]. When the suppressor is inactivated, the levels of thrombospondin mRNA decrease and the amount of secreted protein becomes inadequate to block the angiogenic activity of constitutively produced growth factors (Fig. 1). During in vivo carcinogen-initiated transformation of hamster keratinocytes into carcinomas, it has been shown by cell fusions that a tumor

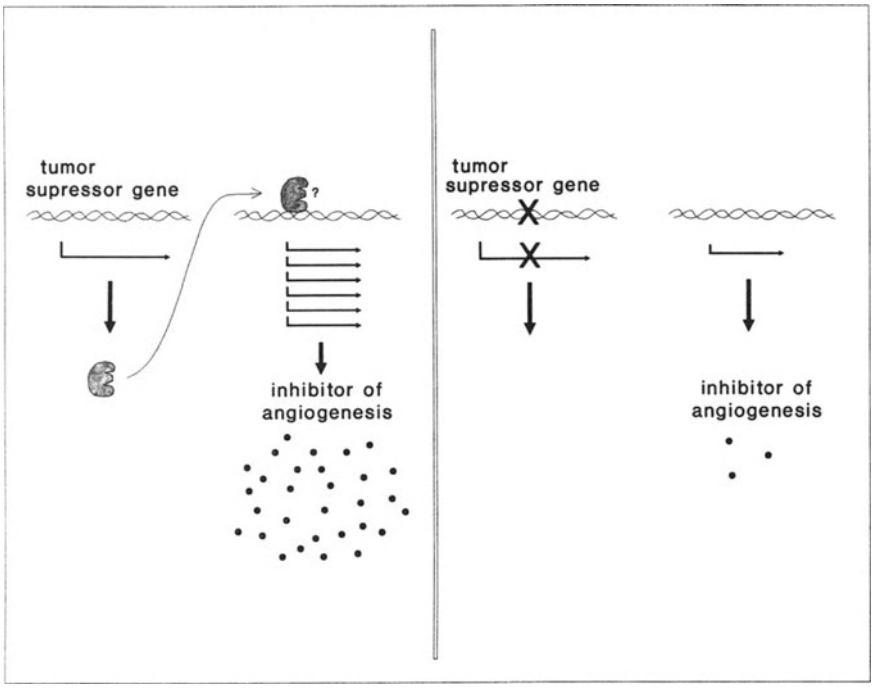

Figure 1. Control of angiogenesis by a tumor suppressor gene. In hamster fibroblasts, an active tumor suppressor gene mediates a high level of transcription from a second gene that encodes an inhibitor of angiogenesis, the matrix protein thrombospondin. Inactivation of the suppressor gene results in reduction in the transcription and secretion of thrombospondin [46,61]. Preliminary experiments indicate that analogous mechanisms may link inhibitors and suppressor genes in human tumors, as returning a suppressor gene to a human neuroblastoma line or to a human osteosarcoma line causes upregulation of the production of a different inhibitor of angiogenesis.

suppressor gene is lost as cells of the developing carcinoma gain the ability to be angiogenic [66].

In humans, the von Recklinghausen neurofibromatosis suppressor gene has been indirectly implicated in angiogenesis in experiments showing that Schwann cells cultured from neurofibromas (assumed to have lost expression of at least one allele of the NF-1 gene) are angiogenic, whereas Schwann cells cultured from normal individuals are not [91]. In two instances where human tumor cell lines have been reverted to nontumorigenic lines by introduction of a suppressor gene (a neuroblastoma line reverted by introduction of normal chromosome 17 [92] and an osteosarcoma line reverted by the cloned retinoblastoma suppressor gene [93]), the suppressed cells begin producing an activity inhibitory for angiogenesis (Tolsma, Huang, Bader, Stanbridge, Polverini, and Bouck, unpublished data). The p53 tumor suppressor gene could also be involved in suppression of angiogenesis, as

both it and the retinoblastoma gene have recently been shown by transient transfection assay to be able to repress the IL-6 promoter, along with promoters of many other genes [94]. Although not yet rigorously tested in vivo, in vitro data suggest that IL-6 is angiogenic [95], thus it is possible that the complete loss of p53, which has been shown to occur in some tumors, permits the increased expression of this putative angiogenic factor.

Future studies and therapeutic options

The above suggestions linking oncogenes and anti-oncogenes to the activation of tumor angiogenesis are tenuous but amenable to experimental testing. Most angiogenic factors and inhibitors are released by cells in culture and can be collected, assayed, purified, and identified, and their contribution to in vivo neovascularization can be assessed. The identification of angiogenic molecules offers insight into the functions of oncogenes and anti-oncogenes by defining molecules crucial to tumorigenicity that they encode or regulate. Inducers of angiogenesis make tempting therapeutic targets; many stimulate the growth of tumor cells as well as of the supporting vessels, and these offer an opportunity to interfere with both tumorigenic actions. In contrast, the inhibitors of angiogenesis are attractive as direct therapeutic agents that are potentially able to block tumor progression and to hold covert metastases in check. Specific inhibitors seem to be lost from different tumors, raising the possibility of being able to treat with the inhibitor produced by cells that are the normal precursors of that particular tumor. Whether such anti-angiogenic therapy is based on natural inhibitors or on some of the many xenobiotics that also block neovascularization, it also provides a theoretical bonus. Inhibitors of neovascularization directly target not the unstable, constantly evolving tumor cell population but rather the activated, normal diploid endothelial cell whose karyotype is stable, whose behavior is predictable, and whose sensitivity should be similar from one individual to another and remain constant over time.

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References

1. Bishop JM: Molecular themes in oncogenesis. Cell 64:235–248, 1991.
2. Klagsbrun M, D'Amore PA: Regulators of angiogenesis. Annu Rev Physiol 53:217–239, 1991.

3. Blood CH, Zetter BR: Tumor interactions with the vasculature: Angiogenesis and tumor metastasis. Biochim Biophys Acta 1032:89–118, 1990.
4. Dunn CJ, Hardee MM, Gibbons AJ, Staite ND, Richard KA: Local pathological responses to slow-release recombinant interleukin-1, interleukin-2 and g-interferon in the mouse and their relevance to chronic inflammatory disease. Clin Sci 76:261–263, 1989.
5. Giulian D, Woodward J, Young DG, Krebs JF, Lachman LB: Interleukin-1 injected into mammalian brain stimulates astrogliosis and neovascularization. J Neurosci 8:2485–2490, 1988.
6. Mahadevan V, Hart IR, Lewis GP: Factors influencing blood supply in wound granuloma quantitated by a new in vivo technique. Cancer Res 49:415–419, 1989.
7. Epstein RJ, Hendricks RL, Stulling RD: Interleukin-2 induces corneal neovascularization in A/J mice. Cornea 9:318–323, 1990.
8. Taniguchi E, Nagae Y, Watanabe H, Ohashi Y, Kinoshita S, Manabe R: The effect of recombinant epidermal growth factor in corneal angiogenesis. Nippon Ganka Gakkai Zasshi 95:52–58, 1991.
9. Fernandez LA, Twickler J, Mead A: Neovascularization produced by angiotensin II. J Lab Clin Med 105:141–145, 1985.
10. Berman M, Winthrop S, Ausprunk D, Rose J, Langer R, Gage J: Plasminogen activator (urokinase) causes vascularization of the cornea. Invest Opthalmol Vis Sci 22:191–199, 1982.
11. Raju KS, Alessandri B, Ziche M, Gullino PM: Ceruloplasmin, copper ions and angiogenesis. J Natl Cancer Inst 69:1183–1188, 1982.
12. Takigawa M, Nishida Y, Suzuki F, Kishi J, Yamashita K, Hayakawa T: Induction of angiogenesis in chick yolk-sac membrane by polyamines and its inhibition by tissue inhibitors of metalloproteinases (TIMP and TIMP-2). Biochem Biophys Res Commun 171:1264–1271, 1990.
13. Ziche M, Morbidelli L, Pacini M, Geppetti P, Alessandri G, Maggi CA: Substance P stimulates neovascularization in vivo and proliferation of cultured endothelial cells. Microvasc Res 40:264–278, 1990.
14. Osthoff KS, Fruhbeis B, Overwien B, Hilbig B, Sorg C: Purification and characterization of a novel human angiogenic factor (h-AF). Biochem Biophys Res Commun 146:945–952, 1987.
15. Fruhbeis B, Zwaldo G, Brockeŕ E, Osthoff KS, Hagemeier H, Popoll H, Sorg C: Immunolocalization of an angiogenic factor (HAF) in normal inflammatory and tumor tissues. Int J Cancer 42:207–212, 1988.
16. McAuslan BR, Reilly WG, Hannan GN, Gole GA: Angiogenic factors and their assay: Activity of formyl methionyl leucyl phenylalanine, adenosine diphosphate, heparin, copper, and bovine endothelium stimulating factor. Microvasc Res 26:323–338, 1983.
17. West DC, Kumar S: Hyaluronan and angiogenesis. In: The Biology of Hyaluronan (Ciba Foundation Symposium 143). Wiley, Chichester, 1989, pp 187–207.
18. Lebel L, Gerdin B: Sodium hyaluronate increases vascular ingrowth in the rabbit ear chamber. Int J Exp Pathol 72:111–118, 1991.
19. Imre G: Lactic acid and neovascularization. Br J Opthalmol 75:254, 1991.
20. Bouck N: Tumor angiogenesis: The role of oncogenes and tumor suppressor genes. Cancer Cells 2:179–185, 1990.
21. Masferrer JL, Rimarachin JA, Gerritsen ME, Falck JR, Yadagiri P, Dunn MW, Laniado-Schwartzman M: 12(R)-hydroxyeicosatrienoic acid, a potent chemotactic and angiogenic factor produced by the cornea. Exp Eye Res 52:417–424, 1991.
22. Dusseau JW, Hutchins PM, Malbasa DS: Stimulation of angiogenesis by adenosine on the chick chorioallantoic membrane. Circ Res 59:163–170, 1986.
23. Dusseau JW, Hutchins PM: Hypoxia-induced angiogenesis in chick chorioallantoic membranes: a role for adenosine. Respi Physiol 71:33–44, 1988.
24. McAuslan BR, Reilly W: Selenium-induced cell migration and proliferation: Relevance to angiogenesis and microangiopathy. Microvasc Res 32:112–120, 1986.

25. Parke A, Bhattacherjee P, Palmer RM, Lazarus NR: Characterization and quantification of copper sulfate-induced vascularization of the rabbit cornea. Am J Pathol 130:173–178, 1988.
26. Weiss JB, Brown RA, Kumar S, Phillips P: An angiogenic factor isolated from tumors: A potent low molecular weight compound. Br J Cancer 40:493–496, 1979.
27. Weiss JB, Hill CR, Davis RJ, McLaughlin B: Activation of mammalian procollagenase and basement membrane-degrading enzymes by a low molecular weight angiogenesis factor. Agents Actions 15:107–108, 1984.
28. Brown RA, Taylor C, McLaughlin B, McFarland CD, Weiss JB, Ali SY: Epiphyseal growth plate cartilage and chondrocytes in mineralising cultures produce a low molecular mass angiogenic procollagenase activator. Bone Miner 3:143–158, 1987.
29. Hockel M, Jung W, Vaupel P, Rabes H, Khaledpour C, Wissler JH: Purified monocyte-derived angiogenic substance (angiotropin) induces controlled angiogenesis associated with regulated tissue proliferation in rabbit skin. J Clin Invest 82:1075–1090, 1988.
30. Oikawa T, Hirotani K, Ogasawara H, Katayama T, Nakamura O, Iwaguchi T, Hiragun A: Inhibition of angiogenesis by vitamin D3 analogues. Eur J Pharm 178:247–250, 1990.
31. Taylor S, Folkman J: Protamine is an inhibitor of angiogenesis. Nature 297:307–312, 1982.
32. Maione TE, Gray GS, Petro J, Hunt AJ, Donner AL, Bauer SI, Carson HF, Sharpe RJ: Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. Science 247:77–79, 1990.
33. Sharpe RJ, Byers HR, Scott CF, Bauer SI, Maione TE: Growth inhibition of murine melanoma and human colon carcinoma by recombinant human platelet factor 4. J Nat Cancer Inst 82:848–853, 1990.
34. Maione TE, Gray GS, Hunt AJ, Sharpe RJ: Inhibition of tumor growth in mice by an analogue of platelet factor 4 that lacks affinity for heparin and retains potent angiostatic activity. Cancer Res. 51:2077–2083, 1991.
35. Andrade SP, Fan TP, Lewis GP: Quantitative in vivo studies on angiogenesis in a rat sponge model. Br J Exp Pathol 68:755–766, 1987.
36. Cozzolino F, Torcia M, Aldinucci D, Ziche M, Almerigogna F, Bani D, Stern DM: Interleukin 1 is an autocrine regulator of human endothelial cell growth. Proc Natl Acad Sci USA 87:6487–6491, 1990.
37. Sidky YA, Borden EC: Inhibition of angiogenesis by interferons: Effects on tumor- and lymphocyte-induced vascular responses. Cancer Res 47:5155–5161, 1987.
38. Arensman RM, Stolar CJH: Vitamin A effect on tumor angiogenesis. J Pediatr Surg 14:809–813, 1979.
39. Oikawa T, Hirotani K, Nakamura O, Shudo K, Hiragun A, Iwaguchi T: A highly potent antiangiogenic activity of retinoids. Cancer Lett 48:157–162, 1989.
40. Ingber D, Folkman J: Inhibition of angiogenesis through modulation of collagen metabolism. Lab Invest 59:44–51, 1988.
41. Tamargo RJ, Leong KW, Brem H: Growth inhibition of 9L glioma using polymers to release heparin and cortisone acetate. J Neuro-Oncol 9:131–138, 1990.
42. Moses MA, Sudhalter J, Langer R: Identification of an inhibitor of neovascularization from cartilage. Science 248:1408–1410, 1990.
43. Shapiro R, Vallee BL: Human placental ribonuclease inhibitor abolishes both angiogenic and ribonucleolytic activities of angiogenin. Proc Natl Acad Sci USA 84:2238–2241, 1987.
44. Grant DS, Tashiro K-I, Segui-Real B, Yamada Y, Martin GR, Kleinman HK: Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. Cell 58:933–943, 1989.
45. Sakamoto N, Iwahana M, Tanaka NG, Osada Y: Inhibition of angiogenesis and tumor growth by a synthetic laminin peptide, CDPGYIGSR-NH2. Cancer Res 51:903–906, 1991.
46. Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, Bouck NP: A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci USA 87:6624–6628, 1990.

47. Vlodavsky I, Michaeli RI, Bar-Ner M, Fridman R, Horowitz AT, Fuks Z, Biran S: Involvement of heparanase in tumor metastasis and angiogenesis. Isr J Med Sci 24:464–470, 1988.
48. Dvorak HF: Tumors: Wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 315:1650–1659, 1986.
49. Folkman J: What is the evidence that tumors are angiogenesis dependent? J Natl Cancer Inst 82:4–6, 1990.
50. Weiss L, Orr FW, Honn KV: Interactions between cancer cells and the microvasculature: A rate-regulator for metastasis. Clin Exp Metastasis 7:127–167, 1989.
51. Weiss L: Metastatic inefficiency. Adv Cancer Res 54:159–211, 1990.
52. Srivastava A, Laidler P, Davies RP, Horgan K, Hughes LE: The prognostic significance of tumor vascularity in intermediate-thickness (0.76–4.0 mm thick) skin melanoma. Am J Pathol 133:419–423, 1988.
53. Schor AM, Schor SL: Tumor angiogenesis. J Pathol 141:385–413, 1983.
54. Folkman J: Tumor angiogenesis. Adv Cancer Res 43:175–203, 1985.
55. Folkman J, Klagsbrun M: Angiogenic factors. Science 235:442–447, 1987.
56. Presta M, Rifkin DB: New aspects of blood vessel growth: Tumor and tissue-derived angiogenesis factors. Haemostasis 18:6–17, 1988.
57. Paweletz N, Knierim M: Tumor-related angiogenesis CRC. Crit Rev Oncol Hematol 9:197–242, 1989.
58. Polverini PJ, Leibovich SJ: Effect of macrophage depletion on growth and neovascularization of hamster buccal pouch carcinomas. J Oral Pathol 16:436–441, 1987.
59. Starkey JR, Crowle PK, Taubenberger S: Mast-cell-deficient W/Wv mice exhibit a decreased rate of tumor angiogenesis. Int J Cancer 42:48–52, 1988.
60. Welch DR, Schissel DJ, Howrey RP, Aeed PA: Tumor-elicited polymorphonuclear cells, in contrast to 'normal' circulating polymorphonuclear cells, stimulate invasive and metastatic potentials of rat mammary adenocarcinoma cells. Proc Natl Acad Sci USA 86:5859–5863, 1989.
61. Rastinejad F, Polverini PJ, Bouck N: Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. Cell 56:345–355, 1989.
62. Briozzo P, Badet J, Capony F, Pieri I, Montcourrier P, Barritault D, Rochefort H: MCF7 mammary cancer cells respond to bFGF and internalize it following its release from extracellular matrix: A permissive role of cathepsin D. Exp Cell Res 194:252–259, 1991.
63. Basset P, Bellocq JP, Wolf C, Stoll I, Hutin P, Limacher JM, Podhajter OL, Chenard MP, Rio MC, Chambon P: A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature 348:699–704, 1990.
64. van den Hooff A: Stromal involvement in malignant growth. Adv Cancer Res 50:159–196, 1988.
65. van den Hooff A: The role of stromal cells in tumor metastasis: A new link. Cancer Cells 3:186–187, 1991.
66. Moroco JR, Solt DB, Polverini PJ: Sequential loss of suppressor genes for three specific functions during in vivo carcinogenesis. Lab Invest 63:298–306, 1990.
67. Heidaran MA, Fleming TP, Bottaro DP, Bell GI, Di Fiore PP, Aaronson SA: Transformation of NIH3T3 fibroblasts by an expression vector for the human epidermal growth factor precursor. Oncogene 5:1265–1270, 1990.
68. Rosenthal A, Lindquist PB, Bringman TS, Goeddel DV, Derynck R: Expression in rat fibroblasts of a human transforming growth factor-α cDNA results in transformation. Cell 46:301–309, 1986.
69. Derynck R: Transforming growth factor α. Cell 54:593–595, 1988.
70. McGeady ML, Kerby S, Shankar V, Ciardiello F, Salomon D, Seidman M: Infection with a TGF-a retroviral vector transforms normal mouse mammary epithelial cells but not normal rat fibroblasts. Oncogene 4:1375–1382, 1989.
71. Thomas KA: Transforming potential of fibroblast growth factor genes. Trends Biochem Sci 13:327–328, 1988.

72. Goldfarb M: The fibroblast growth factor family. Cell Growth Differ 1:439–445, 1990.
73. Kandel J, Bossy-Wetzel E, Radvanyi F, Klagsbrun M, Folkman J, Hanahan D: Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. Cell 66:1095–1104, 1991.
74. Rogelj S, Weinberg RA, Fanning P, Klagsbrun M: Basic fibroblast growth factor fused to a signal peptide transforms cells. Nature 331:173–175, 1988.
75. Kallinowski F, Wilkerson R, Moore R, Strauss W, Vaupel P: Vascularity, perfusion rate and local tissue oxygenation of tumors derived from ras-transformed fibroblasts. Int J Cancer 48:121–127, 1991.
76. Thompson TC, Southgate J, Kitchener G, Land H: Multistage carcinogenesis induced by ras and myc oncogenes in a reconstituted organ. Cell 56:917–930, 1989.
77. Alonso T, Morgan RO, Marvizon JC, Zarbl H, Santos E: Maligant transformation by ras and other oncogenes produces common alterations in inositol phospholipid signaling pathways. Proc Natl Acad Sci USA 85:4271–4275, 1988.
78. Barker K, Aderem A, Hanafusa H: Modulation of arachidonic acid metabolism by Rous sarcoma virus. J Virol 63:2929–2935, 1989.
79. Han J, Sadowski H, Young DA, Macara IG: Persistent induction of cyclooxygenase in p60v-src-transformed 3T3 fibroblasts. Proc Natl Acad Sci USA 87:3373–3377, 1990.
80. Xie W, Chipman JG, Robertson DL, Erikson RL, Simmons DL: Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc Natl Acad Sci USA 88:2692–2696, 1991.
81. Berkenpas MB, Quigley JP: Transformation-dependent activation of urokinase-type plasminogen activator by a plasmin-independent mechanism: Involvement of cell surface membranes. Proc Natl Acad Sci USA 88:7768–7772, 1991.
82. Vogt PK, Bos TJ: jun: Oncogene and transcription factor. Adv Cancer Res 55:1–35, 1990.
83. Schweigerer L, Schwab M, Fotsis T: Endothelial cell growth factors in human neuroblastoma cells transfected with the human mycn oncogene. Abstract 121, International Symposium on Angiogenesis, St. Gallen, Switzerland, 1991.
84. Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY, Hammond D: Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Eng J Med 313:1111–1116, 1985.
85. Montesano R, Pepper MS, Mohle-Steinlein U, Risau W, Wagner EF, Orci L: Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing the middle T oncogene. Cell 62:435–445, 1990.
86. Wagner EF: On transferring genes into stem cells and mice. EMBO J 9:3024–3032, 1990.
87. Folkman J, Watson K, Ingber D, Hanahan D: Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature 339:58–61, 1989.
88. Hanahan D: Dissecting multistep tumorigenesis in transgenic mice. Ann Rev Genet 22:479–519, 1988.
89. Dolberg DS, Hollingsworth R, Hertle M, Bissell MJ: Wounding and its role in RSV-mediated tumor formation. Science 230:676–678, 1985.
90. Schuh AC, Keating SJ, Monteclaro FS, Vogt PK, Breitman ML: Obligatory wounding requirement for tumorigenesis in v-jun transgenic mice. Nature 346:756–760, 1990.
91. Sheela S, Riccardi VM, Ratner N: Angiogenic and invasive properties of neurofibroma Schwann cells. J Cell Biol 111:645–653, 1990.
92. Bader SA, Fasching C, Brodeur GM, Stanbridge EJ: Dissociation of suppression of tumorigenicity and differentiation in vitro effected by transfer of single human chromosomes into human neuroblastoma cells. Cell Growth Differ 2:245–255, 1991.
93. Huang H-JS, Yee J-K, Shew J-Y, Chen P-L, Bookstein R, Friedmann T, Lee EY-HP, Lee W-H: Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. Science 242:1563–1566, 1988.
94. Santhanam U, Ray A, Sehgal PB: Repression of the interleukin 6 gene promoter by p53

and the retinoblastoma susceptibility gene product. Proc Natl Acad Sci USA 88:7605–7609, 1991.
95. Motro B, Itin A, Sachs L, Keshet E: Pattern of interleukin 6 gene expression in vivo suggests a role for this cytokine in angiogenesis. Proc Natl Acad Sci USA 87:3092–3096, 1990.

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