# Perspectives on Clonogenic Tumor Cells, Stem Cells, and Oncogenes<sup>1</sup>

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The last decade has seen major advances in both cellular and molecular aspects of cancer biology. Tissue culture methods that allow the recognition of clonogenic cells within human tumors have been described (37, 64); on the basis of theoretical arguments and the application of these techniques, it has been proposed that neoplasms may be regarded as stem cell systems in which a minority of cells have the proliferative capacity to maintain the tumor whereas the majority of cells demonstrate differentiation features and have limited proliferative potential. This view has been summarized as a "stem cell" model of tumor growth (59) and is consistent with data concerning normal cell renewal (83) and with analysis of kinetics of survival of neoplastic cells following treatment (101).

In parallel with these developments, investigations regarding the molecular biology of neoplasia have shown that aspects of the malignant character of human tumors are governed by the "activation" and/or the inappropriate expression of certain cellular genes that resemble retroviral transforming genes (oncogenes) (reviewed in Ref. 50). Some 20 such genes ("c-onc" genes) have been described; their relationship to carcinogenesis has been attributed to point mutations in a c-onc sequence creating an abnormal gene product or to loss of regulation of transcription of c-onc genes arising through processes such as gene amplification or translocation to a transcriptionally active area of a chromosome. Recently, evidence of relationships between c-onc genes, endogenous peptide growth factors, and the control systems that regulate entry into cell cycle and subsequent proliferative behavior has been presented.

These two sources of information (tumor stem cell organization and oncogene activation) have not previously been integrated. We will first present our perspectives of the anticipated impact of research on clonogenic cells on tumor biology and clinical oncology. Subsequently, we will review certain key observations related to oncogenes and attempt to establish an integration between the concept of cell heterogeneity (imposed by viewing a tumor as a stem cell system) and results showing that c-onc gene transcription is related to cell differentiation and can be modulated by certain growth factors.

# Stem Cell Model of Tissue Maintenance and Tumor Growth

The tumor stem cell concept derives primarily from study of normal renewing cell populations. The tissues comprising such cell populations have a requirement for cell replacement based on the finite life span of functional, differentiated cells. Cell production and cell loss must be balanced to maintain steady state in terms of tissue size. The most studied examples of such cell renewal tissues are bone marrow, intestinal mucosa, and epidermis (53, 83).

A common way in which to represent the properties of a cell renewal system is as a hierarchy of cells with a spectrum of proliferative potential (83). Steady-state control can be based on the premise that proliferative potential and differentiation are opposing processes; *i.e.*, as cells differentiate they lose proliferative potential, such that terminally differentiated cells are proliferatively inert. One diagrammatic representation of this concept is shown in Chart 1.

Cells which have the unique combination of properties of being able to initiate and maintain such clonal hierarchies are termed stem cells; the distinguishing feature of such cells is their capacity for self-renewal. The alternate fate of a stem cell division is the generation of cells committed to differentiate. In the process of clonal expansion to produce functionally differentiated end cells, the committed cells are considered to undergo a number (n) of exponential divisions (clonal expansion number). The cells within this expansion compartment have been termed "transitional cells." The distribution of cells within the system can be treated mathematically by assuming probability functions (59): the PSR<sup>3</sup> and the probability of commitment to differentiate, q = 1 - PSR. In the normal steady-state situation, the number of cells entering the terminally differentiated compartment is equal to the number of differentiated cells lost through senescence, and PSR is close to 0.5.

Stem cells are normally regarded as being a minority subpopulation of cells in any particular tissue. This can be confirmed in circumstances in which the renewal tissue has a recognizable geometry allowing localization of progenitor, transitional, and terminally differentiated cells, *e.g.*, intestinal mucosa, epidermis. For tissues with no organized geometry (*e.g.*, bone marrow), estimates of stem cell frequency have been made indirectly on the basis of developmental assays (64, 107).

# Stem Cells in Human Tumors

Since human tumors arise predominantly in tissues which function as stem cell systems in the normal situation and tumors are tissue specific, it is logical to assume continued stem cell organization in the malignant counterpart (82). Two general theories of tumor origin have been proposed. First, it has long been suggested that a carcinogenic event occurring in a differentiated cell of a particular tissue could render that cell proliferative, although it might retain its ability to organize tissue-specific differentiation. The acquisition of proliferative features in a differ-

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<sup>&</sup>lt;sup>3</sup> The abbreviations used are: PSR, probability of stem cell self-renewal; EGF, epidermal growth factor; AML, acute myeloblastic leukemia; RSV, Rous sarcoma virus; EGFR, epidermal growth factor receptor.



Chart 1. Schematic representation of a stem cell differentiation hierarchy. As cells move down the hierarchy, they progressively lose proliferative potential and gain differentiation characteristics. The resultant end cell population is differentiated but nondividing.

entiated cell necessitates proposing the process of "dedifferentiation."

The second class of theories proposes that tumors arise from carcinogenic events that occur in stem cells of a particular tissue. The proliferative potential and determination in terms of cellular differentiation of this group of cells obviates the need to propose dedifferentiation as a mechanism of tumor growth. Rather, it is necessary to propose only that the changes associated with the carcinogenic insult cause a loss of control and disassociation of the normal stem cell functions, cell renewal and cell differentiation. The ability of the subsequent tumor to organize tissuespecific differentiation (to varying degrees in different tumors) would be a function of the extent to which normal stem cell function had been deranged by the carcinogenic event. One end point of the damage might be a loss of the control of cell renewal and commitment to differentiation such that the tumor tissue grows (PSR > 0.5), rather than maintaining a steady-state equilibrium.

A number of sources of supportive evidence have been proposed in favor of the stem cell origin and growth of human tumors. These are summarized in Table 1. They include the documentation of tissue-specific differentiation in tumor cell populations, the implication of small target size for tumor control with radiotherapy, and the evidence for tumor clonality generated by analysis of glucose-6-phosphate dehydrogenase heterozygotes. Such evidence has generated wide acceptance for a stem cell model of tumor growth for hemopoietic and epithelial tumors. However, there remains considerable doubt as to the applicability of the model to tumors arising in conditionally renewing tissues, such as liver.

#### Assessment of Clonogenic Cells

On the basis of the previous arguments, tumor stem cells are probably responsible *in vivo* for tumor repopulation after therapy and metastatic growth (101). A variety of *in vivo* repopulation procedures (usually dependent on the large proliferative capacity of stem cells) exist in transplantable tumor models for the quantitation of such cells (for a review, see Ref. 101). For human tumors, however, more indirect means must be applied to the study of proliferative cells with potential for repopulation. One of the most valuable and versatile approaches has been the measurement of colony formation by tumor cells in artificial tissue culture environments. Although the ability of a cell to form a colony implies substantial proliferative capacity (at least 6 divisions for a colony of  $\geq$ 50 cells), this does not unambiguously quantitate stem cells. An important distinction must be made between clonogenic cells in tissue culture and those cells responsible *in vivo* for manifestations of tumor growth (99). The elucidation of the relationship between these two cell populations remains a high priority.

Attempts to quantitate clonogenic cells from human tumors have drawn heavily on the experience gained through the study of normal and malignant human hemopoiesis and experimental animal tumors (45, 73, 77, 80, 94). The most popular tissue culture methods for hemopoietic and epithelial malignant cell populations involve growth of cells in medium made semisolid with dilute agar or viscous with methylcellulose. Both environments limit daughter cell migration and prevent anchorage of cells to the surface of the tissue culture vessel.

A number of reports have described specific alterations to colony-forming assays used for the study of normal hemopoietic cells, which allow selective growth of blasts from acute myeloblastic or lymphoblastic leukemia (11, 27, 44, 81). Salmon et al. have developed assays using diluted agar in an enriched medium to support the growth of colony-forming cells in hemopoietic and solid tumors such as myeloma (30, 38), ovarian cancer (39), and melanoma (70). Using similar techniques, a variety of additional solid tumors have been studied, e.g., small cell carcinoma of the lung (15), neuroblastoma (112), colon cancer (49), and transitional cell carcinoma of the bladder (10). Concurrently, Courtenay et al. (20-22, 99) have developed cloning assays from human tumor xenografts or directly from tumors. This method uses replenishable liquid medium over agar; tumor cells are admixed with rat RBC in the agar, and reduced oxygen tension is used during incubation. In parallel studies, this latter procedure gives higher plating efficiency for several tumor types when compared to the assay of Hamburger and Salmon (108).

While colony-forming assays for human hemopoietic tumor cells have invariably required the addition of specific growth factors (usually supplied by conditioned medium), such factors are not absolutely required for growth of colony-forming cells

#### Table 1

Evidence supporting the validity of a stem cell model for human cancer growth

- Tissue-specific call differentiation exists in tumors and in many instances is the basis for histopathological diagnosis.
- Within a given histological tumor type, there appears to be an inverse relationship between indices of proliferation (mitotic rate or thymidine-labeling index) and the degree of tumor cell differentiation (tumor grade) (67, 82, 101).
- 3. Clinical experience with radiation therapy suggests that only a small proportion of tumor cells must be killed in order to abolish tumor regeneration. Permanent local control of some basal cell carcinomas can be effected with low doese of radiation (12), implying that the regenerating cell (stem cell) fraction of these tumors is small.
- In existing clonogenic assays, the number of colonies generated from suspensions of cells from hemopoletic and epithelial human tumors is usually in the range of 0.001 to 1.0% of the total cells (91).
- Fractionation of suspensions of cells from human tumors has demonstrated that proliferative activity, clonogenic potential, self-renewal capacity, and cell differentiation features are restricted to separate subpopulations with defined physical properties (2, 58, 60, 64).
- 6. In certain hematological tumors such as chronic myelogenous leukemias, clonal markers, such as the Philadelphia chromosome and the isoenzymes of glucose-6-phosphate dehydrogenase (32), have been found in multiple cell lineages. This is consistent with the origin of the tumor in a multipotential stem cell, although the majority of cells within the tumor consist of differentiated progeny.

from many solid tumors. There are, however, recent reports of modulation of the growth of clonogenic cells from solid tumors with hormonal additives of physiological relevance, including EGF (41, 68, 69, 71). It has also been noted that the growth of colonies from a number of epithelial tumors seems to be influenced by factors released from coexisting macrophage-like cells (7, 39, 40). These findings indicate that improved plating efficiencies may be achieved by the use of tissue-specific growth factors that stimulate cell proliferation, and they emphasize that plating efficiency may vary not only with the proliferative potential of cells but also with the particular mitogenic stimuli provided. In addition, the data indicate that the phenotype of clonogenic cells may include expression of receptors for various hormones which are known to control proliferation in the normal tissue equivalents.

From a practical point of view, "clonogenicity" has been defined variously under different circumstances to be growth of individual cells to clones of  $\geq$ 20 cells up to  $\geq$ 64 cells. Using these criteria, estimates have been made of the frequency of clonogenic cells in human tumors. Such estimates range from 0.001 to 1.0% of tumor cells; the biological significance of this variation is not established.

Even though plating efficiency is probably not optimal with existing assays, the properties of clonogenic cells are understood to some degree, using the assumption that those cells which do grow are representative of the total with growth potential. For example, the proportion of clonogenic cells found to be in active cycle (by [<sup>3</sup>H]thymidine suicide assays) has been found to be very high (40 to 80%) in myeloma, AML, and ovarian carcinoma (39, 72, 98).

The physical properties of clonogenic tumor cells are also known to some degree. They can be enriched in some tumor populations using physical fractionation procedures (38, 58, 60, 64) and, within a particular tumor type, the physical characteristics of the clonogenic tumor cells are similar in different patients.

# **Relationship between Clonogenic Cells and Stem Cells**

It has been recognized that assessment of colony formation in culture by individual tumor cells (hemopoietic or epithelial) does not necessarily quantitate tumor stem cells (4, 101). In the absence of an *in vivo* assay for tumor regeneration (as has been possible in transplantable animal tumor models), indirect methods have been used to probe the relationship between clonogenic tumor cells and tumor stem cells.

For example, it has been found that a proportion of tumor colonies derived from small cell carcinomas of the lung have the proliferative capacity to form tumors in immune-privileged sites in nude mice (14). Colony-replating assays to assess the property of self-renewal have also proved useful in this regard. It has been shown that a small and variable proportion of colonies derived from AML (9), ovarian carcinoma (18), and melanoma (106) have the capacity to regrow when disaggregated and replated. Furthermore, the quantitation of this property has led to the conclusion that the capacity for self-renewal may be a biological property of importance in determining prognosis in AML (65). Recently, colony size has been proposed as a means of determining which clonogenic cells have stem cell properties (59). It has been noted that, in ovarian carcinoma, the clonogenic cells with the greatest proliferative capacity, as judged by colony size, are likely to display stem cell properties (5).

# Perspectives on the Impact of Human Tumor Clonogenic Assays

The impact of clonogenic assays has been most evident in three areas: description of tumor cell heterogeneity with respect to differentiation; studies of biological control of tumor cell proliferation; and chemotherapeutic research for identification of new agents or prediction of patient response to existing agents.

The concept that human tumor cells may be heterogeneous with respect to proliferative potential has drawn support from the recent studies of tissue culture clonogenic potential. Although accurate quantitation of such potential is currently limited, the basic premise is unlikely to be false. A "stem cell differentiation model of tumor growth" has been proposed to explain such heterogeneity (59). Tumors are considered to be "caricatures" of their normal tissue renewal counterparts (82). The most important corollary to this view is that curative tumor therapy must be aimed at the stem cell population and, conversely, that the fate of the majority of the cells within a tumor is of no consequence to long-term tumor growth. This provides a pressing rationale for further attempts to understand the biological control processes acting at the tumor stem cell level.

Clonogenic assays have been used to determine the phenotype representative of primitive cells within a tumor. "Suicide" or sorting techniques can be applied to determine if clonogenic cells display particular markers of differentiation. Alternatively, analysis can be made of the concordance of cell differentiation and clonogenic phenotypes after cell fractionation on physical parameters (2, 5, 60). Experiments such as these may eventually lead to an understanding of the *in vivo* generation of cell differentiation heterogeneity. Additionally, it is hoped that knowledge of features such as the cell surface phenotype of the stem cell population may allow the development of novel therapeutic or diagnostic/prognostic approaches.

If the stem cell model is valid, it seems intuitive that any measure that could decrease the PSR of tumor stem cells, even if nonlethal to these cells, would be of benefit clinically, Indeed, computer simulation systems can be used to show that even small changes in PSR might have significant impact on tumor burden (33, 59). However, our knowledge of the factors influencing cell renewal and differentiation of tumor stem cells is severely limited. In AML, the renewal potential of progenitor cells has been found to be decreased by exposure to interferon (105) or to low doses of  $1-\beta$ -D-arabinofuranosylcytosine (6). A few reports in the recent literature suggest that some solid tumor clonogenic cells may bear receptors for peptide hormones (41, 69). If neoplastic stem cells retain any sensitivity, even at pharmacological doses, to the factors that normally regulate their proliferative behavior, efforts to exploit this property, perhaps in combination with cytotoxic therapy, would be warranted. As we shall show. studies of inducers and repressors of c-onc gene transcription may provide clues as to specific substances that might be relevant in this regard.

A major clinical role has been proposed for clonogenic assays in aiding in the prediction of individual patient response to chemotherapy. This approach is predicated on the notion that cells having a large proliferative capacity may be important in determining growth properties of the tumor and that the predictive value of clonogenic assays would be much greater than the knowledge of nonspecific proliferative characteristics (e.g., [<sup>3</sup>H]thymidine incorporation). Early observations, based on retrospective and prospective analysis of patients treated independently of in vitro test results, are encouraging (92, 110, 111), although it must be noted that similar retrospective correlations have been reported over the last few decades using less sophisticated end points of tumor cell proliferation. Major practical limitations exist with respect to accurately measuring in vitro drug effects on the clonogenic subpopulation (93). The concept is now being tested in randomized clinical trials to attempt to detect patient benefit (115); the future development of "individualized chemotherapy" will depend on the result of these trials. At the moment, it is possible to attempt such trials in tumor types amenable to quantitative studies; the tumors which most closely approach this situation are melanoma, ovarian carcinoma, and certain leukemias. Another area of chemotherapeutic research in which clonogenic assays may have direct benefit is in new drug screening. Attempts are under way to assess the integration of such procedures into the current preclinical drug screens.4

Finally, the heterogeneity of proliferative behavior which forms the basis of the stem cell model of cellular proliferation may have important implications for studies relating gene expression to proliferative potential. Such a model would predict that cells derived from a primary tissue, whether normal or neoplastic, will be heterogeneous with respect to the expression of genes that regulate proliferation. The second part of this paper will deal with the evidence implicating cellular oncogene expression in such regulation. An important difference between normal and neoplastic stem cells may be the failure of the control systems that normally regulate expression of these genes in the latter case. The techniques developed to probe tumor cell clonogenicity and differentiation may allow clarification of this issue for human tumors.

#### **Oncogenes: Current Concepts**

Two lines of investigation have contributed to our current conception of oncogenes as genes that are important not only in oncogenesis but also in the regulation of normal cellular proliferation. The first of these, the study of tumor retroviruses, originated with the characterization of RSV (89). Studies of RSV mutants that are either temperature sensitive for (61) or completely lacking in (113) transforming ability led to the identification of a segment of the RSV genome that is both necessary and sufficient for the transformation of infected cells (29). This seqment, known as the src gene, was the first oncogene to be characterized. Huebner and Todaro (43) had previously speculated that, if viral genes could induce certain tumors, perhaps other, nonvirally induced neoplasms could be caused by the derepression of similar viral genes that had been incorporated into the genome of higher organisms through the course of evolution. When Stehelin et al. (102) tested this hypothesis using a radiolabeled src gene as a probe to scan the DNA of normal chickens, they indeed found a similar gene. This gene, named csrc, coded for an enzyme with activity similar to that of the src product, and it was subsequently shown (19, 78) that c-src is not only present but also transcribed at low levels in normal cells of vertebrates and lower organisms. More than a dozen viral oncogenes have subsequently been characterized, and each has been found to be homologous to a so-called cellular oncogene (*c-onc* gene) found in the normal genome of a wide variety of species. However, it is now believed, on the basis of analysis of the structure of the oncogenes, that they arose early in evolution, that they have been conserved, and that the rapidly transforming tumor viruses have only recently acquired copies from the genome of infected cells.

A separate line of investigation has led investigators to the same cellular transforming genes. In 1979, it was shown (96) that DNA prepared from chemically transformed rodent cell lines could, in DNA transfer experiments, confer a transformed phenotype to recipient NIH3T3 cells and that the phenomenon could be observed even when tissue and/or species barriers were crossed (48, 95). In 1982, Shih and Weinberg (97) identified a single gene present in the DNA of a human bladder tumor cell line that was responsible for NIH3T3 transformation in DNA transfer experiments, and it was demonstrated (26, 79) that the transforming gene active in this bladder tumor cell line (and other similar lines) was homologous to the transforming gene of the Harvey sarcoma virus.

It has been shown that the protein products of the oncogenes are heterogeneous with respect to function and intracellular localization. Many are membrane-bound protein kinases; others are nuclear in location. In certain cases, specifically characterized mutations have given rise to subtle differences between c-onc genes and their transforming counterparts, and this has led to the "mutational hypothesis," *i.e.*, that the "activation" of a c-onc gene to a *bona fide* transforming gene results from mutational events (87, 104). Activated oncogenes have now been detected in a wide variety of tumors and neoplastic cell lines, such as colon, breast, lung, and pancreas (56, 63, 84, 103). It has been noted that these oncogenes are not tissue specific; an activated H-ras oncogene, for example, appears to be present in solid tumors from a number of different sites.

In contrast, the "dosage hypothesis" supposes that the crucial difference between normal c-onc genes and transforming genes concerns the level of expression. Regulated c-onc expression is postulated to be a normal event, perhaps related to the control of cellular proliferation, while neoplasia is postulated to result from unregulated transcription of the very same genes. Evidence consistent with the dosage hypothesis comes from work demonstrating that transformation can be induced by c-onc genes in cases where expression of the gene is increased by changes in promoter or regulator sequences, by amplification, or by translocation to an area of active transcription (Refs. 17 and 25; reviewed in Ref. 50). The mutational and dosage mechanisms of c-onc activation are not, of course, mutually exclusive.

## **Complementation Groups of Oncogenes**

Studies of DNA tumor viruses that are capable of transforming human fibroblasts grown in primary culture have revealed that transformation is dependent, in certain cases, on the expression of at least 2 viral genes (42, 86, 109). Human adenovirus, for example, must express both the E1a and E1b regions of its genome to effect transformation. It has been shown that the E1a product confers an "immortalizing" or "establishment" phenotype, causing primary cultures to grow indefinitely. On the other hand, it is the E1b product that appears to confer to immortalized fibroblasts certain other phenotypic changes associated with

<sup>&</sup>lt;sup>4</sup> R. H. Shoemaker, M. K. Wolpert-DeFilippes, D. H. Kern, M. M. Lieber, R. W. Makuch, N. R. Melnick, W. T. Miller, S. E. Salmon, R. M. Simon, J. M. Venditti, and D. D. Von Hoff. Application of a human tumor colony forming assay to new drug screening, submitted for publication.

cancer, such as tumorigenicity, anchorage-independent growth, and rapid proliferation. Experiments at the Cold Spring Harbor Laboratory (90) and the Massachusetts Institute of Technology (51), originally designed to assess the ability of oncogenes to transform primary fibroblast cultures rather than the NIH3T3 immortalized cell line, have shown that certain oncogenes seem to have "E1a-like" functions and that certain others have "E1blike" functions.

It was observed that no single oncogene tested, regardless of its efficiency at transforming NIH3T3 cells, could transform fibroblasts from primary cultures. However, cotransfection with certain pairs of genes, such as ras gene and a myc gene or a ras gene and an E1a-region gene, was effective in transforming primary fibroblasts. These experiments have led to the division of certain oncogenes and DNA virus genes into 2 functionally distinct "complementation groups." One (Group A) is concerned with immortalization, and the other (Group B) appears to be related to other aspects of the malignant phenotype. It has recently been shown that the proteins coded by certain Group A oncogenes are found in the nucleus and show a degree of structural homology to each other (85). Group B oncogene products have not been shown to be homologous to the proteins encoded by Group A oncogenes and often are cytoplasmic in location. Many oncogenes have not been assigned to either complementation group; it is possible that further investigation may allow the classification of these genes to Group A, Group B, or as yet undefined functional categories of oncogenes. A simplified summary of the effect of the genes of Complementation Groups A and B is presented in Table 2.

# **Function of Cellular Oncogenes**

There are several lines of evidence that make it reasonable to speculate that c-onc genes are, under normal circumstances, involved in the regulation of cellular proliferation. The high degree of conservation of these genes throughout evolution is consistent with the view that they are involved in an essential cellular process; certain human cellular oncogenes have sequences related to genes involved in cell cycle control in yeast (57). An initially unexpected observation was the remarkable similarity between the src gene product and the intracellular enzymes that mediate the effects of certain potent mitogens, such as EGF (reviewed in Ref. 31). This provides a rationale for speculation that the mitogenic effect of substances such as EGF may involve regulation of c-onc transcription. Further evidence relating c-onc genes to the control of normal cellular proliferation comes from the demonstration that expression of a c-ras oncogene is temporarily elevated following the induction of compensatory hepatocyte proliferation by partial hepatectomy in rats (34) and that certain c-onc genes are expressed at high levels at early periods of murine development (74), when overall rates of proliferation

Table 2			
Effect of transfection of fibroblasts on oncogenes of Complementation Groups A			
and B (see text)			

Effect of	on	is
Group A	Primary cells	Immortalize or no effect
Group A	Cell line	No effect
Group B	Primary cells	Transient proliferation prior to cell death
Group B	Cell line	Transformation or no effect
Both	Primary cells	Transformation
Both	Cell line	Transformation

are higher than in adult organisms.

Of particular relevance to the stem cell model of proliferation are the observations that proteins related to c-onc genes are not homogeneously distributed in all cells of a renewing tissue such as gut or marrow but can be shown to vary from cell to cell according to proliferative and differentiation state. For example, the expression of genes homologous to the transforming sequence of avian myeloblastosis virus and avian erythroblastosis virus has been shown to vary with the stage of differentiation of human and avian hematopoietic cells, respectively (18, 116).

The hypothesis that the normal function of *c-onc* genes is related to control of proliferative behavior is further supported by recent observations concerning the *erb-b* (28) and *sis* (114) genes. The product of the former appears to be homologous to the cell surface EGFR, and the product of the latter is almost identical to platelet-derived growth factor; it is clear that these are genes that code for proteins directly involved in the normal control of cell proliferation by endogenous mitogens.

Further studies are clearly required to test the hypothesis that the heterogeneity of proliferative potential of primary cells from normal and neoplastic tissue is correlated with a heterogeneity of expression of c-onc genes. Development of specific antibodies to c-onc gene products and/or refinements of *in situ* molecular hybridization techniques may permit direct studies of oncogene expression in the individual cells of normal tissues and neoplasms (18). In tissues such as epidermis or intestinal mucosa, in which the location of stem cells relative to differentiated cells is known, this approach may demonstrate stem cell-specific patterns of conc gene expression.

# **Growth Factors Controlling Stem Cell Proliferation**

It is likely that normal stem cell proliferation is regulated by a balance of endogenous proliferation and differentiation-inducing factors. Genes that code for these factors, their receptors, and the intracellular proteins involved in the mediation of their effects might have the "transforming" properties of activated oncogenes if they were altered by mutation and/or inappropriately expressed. Table 3 lists abnormalities of hypothetical proteins involved in the control of proliferation by endogenous mitogens that could lead to uncontrolled cell division.

In prokaryotes, control of proliferation is unrelated to the process of differentiation, and all cells have proliferative potential. As we have shown, this is not the case in tissues of higher organisms, where regulation of proliferation is closely related to the process of differentiation and where indefinite proliferative

Table 3	
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Abnormalities of hypothetical c-onc gene products that could lead to uncontrolled proliferation

Excess production of an endogenous mitogen.

Mutation of gene coding for endogenous mitogen, leading to abnormally high agonist activity of gene product.

Excess production of mitogen receptors.

Mutation of gene coding for receptor leading to abnormally high receptor/mitogen avidity.

Mutation of receptor-linked enzyme such that it is active in absence of mitogen binding.

Excess substrate for receptor-linked enzyme.

Mutation such that substrate for receptor-linked enzyme no longer requires activation (e.g., phosphorylation) to be active.

Mutation such that substrate not readily degradable from activated (phosphorylated) state.

Mutation rendering deactivation enzymes ineffective.

Inadequate production of deactivation enzymes.

capacity has been restricted to stem cells. The more complex control systems required for regulation of tissue renewal in higher organisms necessitate the loss of autonomy of proliferationcompetent cells; this can be accomplished by mechanisms that make cell division conditional on the presence of growth factors normally produced by other cells. Abnormalities leading to the constitutive expression of genes coding for growth factors by proliferation-competent cells that are normally the targets of growth factor action could lead to an autostimulatory mechanism of neoplasia such as that proposed by Sporn and Todaro (100). The control of stem cell proliferation probably is achieved by a complex interaction between several growth factor-receptor systems; there is evidence that cell division may be maximally stimulated by the presence of two or more synergistic growth factors (24).

Rapidly emerging data concerning the regulation of the mvc and erb-b genes may contribute to our understanding of control of cellular proliferation. It has been shown that extracellular growth factors can regulate myc expression. Expression of this gene seems to be correlated with proliferative status and, while it appears to be regulated by a variety of extracellular substances in nontransformed lines (13, 47), it apparently is expressed constitutively at high levels in some transformed cell lines (13). The induction of myc expression in lymphocytes and 3T3 cells by platelet-derived growth factor (47), itself homologous to the product of the sis oncogene, is consistent with the regulation of cell division by the interaction of various c-onc gene-encoded proteins. Reports showing that myc expression can be suppressed, as well as induced, by extracellular factors [e.g., effect of vitamin D on HL-60 cells (88)] suggest that c-onc expression, and hence proliferative behavior, might be governed by a balance of opposing proliferation and differentiation signals present in the microenvironment of the stem cells of a tissue.

In view of accumulating evidence (28, 66) that the erb-b gene codes for a segment of the EGFR protein, this receptor may be a convenient cell surface marker of expression of a c-onc gene, although EGFR levels detectable by radioreceptor assays or monoclonal antibodies vary not only with gene expression but also with factors such as agonist binding and rate of internalization of the receptor from the cell surface (reviewed in Ref. 36). It is of interest that radioautography using radiolabeled EGF has shown that the cells of normal rat epidermis exhibit nonhomogeneous expression of EGFRs, with significantly greater binding of the labeled ligand occurring on the basal cells which are known to be responsible for epidermal renewal (35, 52, 76). Similarly, a decrease in EGFR expression has been shown to accompany terminal differentiation of mouse myoblasts (55) and neuroblastoma cells (75). It is possible that the presence of mitogen receptors may serve as a useful marker of proliferationcompetent cells in these and other tissues. However, it appears that EGFRs, although linked to intracellular mediators leading to mitosis in stem cells, can be linked to other differentiation-specific functions in differentiated cells. The induction of prolactin synthesis in pituitary cells by EGF (46) may be an example of this process.

Several other observations concerning the EGFR are of interest in connection with the status of this protein as the putative product of the *erb-b* oncogene. Certain transformed cell lines produce conditioned media that are highly mitogenic to nontransformed cell lines; these media have been shown to contain, in addition to peptides that act as EGFR agonists ( $\alpha$ -transforming growth factors), others ( $\beta$ -transforming growth factors) that appear to act as inducers of the EGFR (1). The synergistic nature of this combination of effects may explain the potency of such conditioned media in eliciting mitogenic responses. The significance of the high levels of EGFR recently observed in certain human tumors (54) remains to be clarified. If the binding of endogenous mitogens to these receptors is necessary for neoplastic growth, blocking antibodies such as that described by Carpenter *et al.* (16) might decrease tumor cell proliferation. This kind of effect has recently been demonstrated in an *in vivo* nude mouse model (62).

Most attempts to study induction of c-onc gene expression by specific growth factors have relied on the use of immortalized cell lines. The cells of a given line are relatively homogeneous with respect to their response to a growth factor under specified conditions. This is not likely to be the case in primary cells derived from normal tissues or neoplasms. In these populations, there may be considerable heterogeneity of response of individual cells to growth factors. Terminally differentiated cells are not capable of dividing, even under optimum mitogenic stimulus, whereas stem cells can initiate a clone under appropriate conditions. Further studies are required to characterize the heterogeneity of responsiveness of primary cells to the various growth factors that have been shown to induce c-onc expression and proliferation in cell lines.

# Proliferative Behavior in Terms of the Stern Cell Model: Proposed Correlations with Cellular Oncogene Expression

As we have described, the stem cell model postulates two degrees of freedom for stem cells: (a) they may rest or proliferate; and (b) if they proliferate, they may give rise to daughter cells programmed to differentiate with loss of proliferative potential or to daughter cells that fail to differentiate but retain self-renewal capacity (Chart 1). The former relates to regulation of cell cycle entry, while the latter relates to control of differentiation and is related to PSR. In this section, we will summarize evidence that provides a rationale for associating control of cellular oncogene expression with control of PSR and cell cycle entry.

The markedly different proliferative behavior of primary cells and immortalized lines is regarded, in terms of the stem cell model, as resulting from a loss of regulation of stem cell commitment. PSR is thought to approach 1 in immortalized lines, resulting in a population of close to 100% stem cells. This is consistent with the facts that a large majority of cells taken from an immortalized line have self-renewal capacity and that they display much less heterogeneity of differentiation markers than do cells taken from primary normal or neoplastic tissues (Chart 2).

As we have seen, experiments studying the induction of the malignant phenotype by gene transfer to fibroblasts derived from normal tissue, as compared to fibroblasts derived from an immortalized cell line, have demonstrated a clear requirement in the former case for transfer of an "immortalizing gene" in addition to an oncogene from Group B. It is possible to speculate that there is a relationship between the proposed function of "immortalizing" genes in these experiments, and the concept of self-renewal that is derived from the stem cell model. The "immortalization" and relative homogeneity of cells of established lines, as compared to primary cells, may be explained in terms of the stem cell model as consequences of a loss of regulation, in the



Chart 2. Proportion of stem cells (shaded area) typically present in populations of cells taken from normal tissue (A), immortalized lines (B), and neoplastic tissue (C) and typical proliferative behavior of stem cells in each instance. Relative thickness of arrows represents renewal and differentiation probabilities. Note that in A and C the exact proportion of stem cells is tissue (or tumor) specific.



(i) Normal stem cell : PSR controlling genes subject to regulation



(ii) Cell from immortalized line : PSR-controlling genes constitutively expressed ; PSR = I



 (iii) Neoplastic stem cell : PSR - controlling genes (as well as cell cycle controlling genes) inappropriately expressed, and not appropriately modulated by growth stimulating or inhibiting factors.

Chart 3. Proposed control of PSR by immortalizing genes in stem cells from normal renewal tissue (/), stem cells from an immortalized line (//), and stem cells from neoplastic tissue (//). Relative thickness of arrows represents renewal and differentiation probabilities. Normally, PSR is closely modulated according to physiological needs by growth factors that can temporarily increase or decrease transcription of a cellular immortalizing gene. In immortalized line, expression of such genes is constitutive; PSR approaches 1, and this leads to an absence of differentiated cells. In neoplastic stem cells, although PSR is <1, it is no longer under appropriate physiological control. This, together with loss of regulation of cell cycle entry, leads to both uncontrolled proliferation and heterogeneity of differentiation (see text).

former case, of genes that normally are involved in the control of PSR. Thus, the normal function of the cellular homologues of the "immortalizing" complementation group of oncogenes might be related to PSR modulation; transcription of these genes would normally be closely regulated by the growth factors that influence stem cell proliferation. Chart 3 illustrates the integration of these concepts in stem cells from normal tissue, neoplastic tissue, and immortalized lines.

While most immortalized cell lines cannot differentiate, there are several examples where differentiation can be induced, and these provide opportunities to study cellular oncogene expression as it relates to control of PSR. The human leukemia cell line HL-60 has been shown to differentiate under the influence of a variety of specific growth-controlling factors, and this process has been correlated with a decline in *myc* expression (88, 117). It is possible, in keeping with our speculation, that the retinoid or vitamin D-induced suppression of *myc* expression seen in HL-60 cells has the effect of lowering PSR and hence facilitating differentiation. In similar systems, induction of differentiation in M1-1 leukemic cells (23) and F9 murine teratocarcinoma cells (13) has been associated with decreased expression of *c-myb* and *c-myc* genes, respectively.

Recent work has shown that increased transcription of a src gene occurs in cultured mouse hematopoietic cells following infection with a src-containing virus and that this is associated with an increased proportion of "primitive" cells, improved plating efficiency, and enhanced ability to reconstitute the hematopoietic system of lethally irradiated mice (but not with leukemic transformation) (3). These effects are all in keeping with an increase (but not a total loss of control) of PSR in the infected cells, and they emphasize the utility of the stem cell model in studies relating gene expression to proliferative behavior. It is noteworthy that the src gene, which has not been assigned to either complementation group on the basis of previous fibroblast transfection studies, appears to be capable of influencing PSR under certain circumstances. It is possible that the oncogenes that have been shown to complement the *ras* gene in transformation of rat fibroblasts represent a subset of a larger functional group of genes that can influence PSR.

The stem cell model views the control of PSR as distinct from the control of cell cycle entry; i.e., related but distinct systems may determine on the one hand whether a stem cell will rest or divide and on the other hand whether a stem cell division will lead to a clone of cells destined to differentiate or to additional proliferation-competent cells. The control of proliferation in nondifferentiating immortalized cell lines is consistent with this view. As we have seen, immortalized lines may be regarded as having PSR constitutively high, rather than closely regulated, leading to the absence of differentiated progeny. However, it is well known that a variety of specific growth factors may control the rate of proliferation of immortalized cells, despite their constitutively high PSR. Recent work has shown that certain cellular oncogenes are induced by these proliferation-stimulating growth factors (47). It should be emphasized that experimental systems using immortalized cell lines to study control of cell cycle entry represent useful simplifications of in vivo proliferation, because the differentiation option of stem cells does not exist for cells from most immortalized lines.

#### Conclusion

In summary, we have presented evidence which is consistent with the following proposals. Control of cell proliferation and differentiation in renewing tissues depends both on control of cell cycle entry and on the probability of stem cell self-renewal. The phenomenon of immortalization may represent a derangement of the regulation of self-renewal through inappropriate conc expression, leading to a homogeneous nondifferentiating population of stem cells that may retain some control mechanisms regulating entry into cell cycle. Neoplasia, in contrast, may represent a more complex loss of regulation of both cell cycle entry and PSR, resulting in a heterogeneous population of cells showing varying degrees of differentiation.

We have suggested, from an analysis of the literature, that the products of certain cellular oncogenes may control the process of self-renewal and cell cycle entry and that expression of these genes is normally dependent on the presence of appropriate extracellular growth factors. Mutation and/or inappropriate expression of these genes in malignant stem cells might underlie the lack of regulation of these processes evident in tumors. Thus, study of the heterogeneity of *c-onc* expression in the cells constituting renewal tissues may allow an understanding of the molecular mechanisms controlling proliferation and differentiation, and their derangements in neoplasia.

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