A cell cycle hypothesis of cooperative oncogenesis (Review)

BÉNÉDICTE DELAVAL[1] and DANIEL BIRNBAUM

Centre de Recherche en Cancérologie de Marseille, Laboratoire d'Oncologie Moléculaire, UMR599 Inserm et Institut Paoli-Calmettes, 27 Bd. Leï Roure, 13009 Marseille, France

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Abstract. The development of cancer is a multistep process. To understand oncogenesis and adapt appropriate treatments it is important to have a better definition of a number of factors, including the number and order of oncogenic steps, the identity of the targeted cells and deregulated cellular components, and the genes and pathways altered at each step. We propose here a hypothesis of oncogenesis based on the targeting of the cell cycle in two major steps. Oncogenic hits may occur in two sequences: in one scenario a first oncogenic hit alters the regulation of the G1 phase of the cell cycle leading to a proliferative, premalignant syndrome; oncogenesis is completed when a second oncogenic hit relieves the checkpoints of the late phases of the cell cycle. Alternatively, a genetic alteration may hit the late phases first; this leads to a premalignant disease with signs of senescence. In this scenario, the second hit targets the G1 phase. In the two sequences, oncogenesis is based on the cooperation of two hits targeting different phases of the cell cycle and relieving major checkpoints. Stem cells and progenitor cells of various tissues may be variably sensitive to these hits.

Contents

Correspondence to: Dr D. Birnbaum, Centre de Recherche en Cancérologie de Marseille, Laboratoire d'Oncologie Moléculaire, UMR599 INSERM, 27 Bd. Leï Roure, 13009 Marseille, France
E-mail: birnbaum@marseille.inserm.fr

Present address: [1]University of Massachusetts Medical School/ Biotech 2373 Plantation Street, Suite 206, Worcester, MA 01605, USA

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1. Introduction

The malignant cells that fuel a tumor mass have acquired a number of genetic and functional alterations that allow them to survive, grow autonomously, proliferate permanently and disseminate. Epidemiological, mathematical, histoclinical, molecular and experimental data (1-5) suggest that the acquisition of genetic alterations, and subsequent modifications of functional processes, is progressive and cumulative. In 1997, Kinzler and Vogelstein (6) proposed the gatekeeper/caretaker theory to describe the pathways to oncogenesis. Gatekeepers are genes that function in cellular checkpoints to inhibit growth and promote apoptosis in specific conditions. In 2000, Hanahan and Weinberg (7) and Hahn and Weinberg (8) proposed that alterations of functional processes are limited in number and common to all types of cancer cells. To better delineate the multistep hypothesis of cancer, they defined a set of six acquired alterations or traits as necessary and sufficient for all cancer cells. We here propose a model in two main steps associated with cell cycle deregulations. Each step overcomes a cell cycle checkpoint or gatekeeper, allowing continuous cell proliferation. At each step a subset of the six previously defined processes (7,8) is altered. The first step does not induce a full malignant phenotype but a premalignant stage. Genomic instability may or may not be present at the initial step.

2. Multistep oncogenesis in experimental models: Cooperation of oncogenes to transform primary cells

In the early eighties, focus formation in NIH3T3 fibroblast cell line was routinely used in research laboratories to assay the transforming capacity of transfected DNA and isolate new oncogenes. The NIH3T3 established mouse cell line was transformed by a single oncogene such as mutated *RAS*. Two studies showed that, in contrast, not one but two oncogenes were necessary to transform primary cells such as rat embryo fibroblasts (REF) (9,10) or baby rat kidney cells (11). One of the pair of genes had to belong to an 'immortalizing' class of oncogenes, such as *MYC* or adenovirus E1A, and the other to a 'transforming' class of oncogenes, such as mutated *RAS*. Cooperation of the two classes was observed in subsequent studies, and a first coarse classification of oncogenes was established (10). Among 'immortalizing' oncogenes cooperating with *RAS* were *BCL2* (12), *JUN* (13), *D cyclins* (14,15) and E6/E7 open reading frames from human papilloma virus (HPV) 16 (16). Immortalizing oncogenes had no effect on

established cell lines such as NIH3T3, which had already reached a first stage of transformation with the acquisition of an infinite lifespan. In contrast, transforming oncogenes induced anchorage-independent growth and abrogated contact inhibition. Cooperation of oncogenes was also observed in the study of viruses such as avian MH2 (17) or erythroblastosis (18) retroviruses. These experiments paved the way for the multistep theory of oncogenesis (10). They also showed the importance of a first step of cell immortalization and sustained proliferation on the way to cancer. This step is not necessarily associated with genome instability.

Confirmation of a cooperating effect between two classes of oncogenes was further provided by studies of transgenic mice (19,20). Hyperplasia of the mammary gland is the first consequence of the overexpression in this organ of oncogenes of the first class, such as *cyclin D1* (21), *cyclin E* (22) and *CDC25B* (23). Adenocarcinomas occur after a variable latency period and secondary alterations (24) or cooperation with other oncogenes.

More recently, transformation of primary human cells provided important new information on the multistep oncogenic process (8). In contrast to rodent cells, transformation of primary human epithelial and fibroblastic cells requires the combined effect of three proteins, namely SV40 large-T antigen, telomerase catalytic subunit TERT, and mutated RAS (25). Immortalization is obtained by the cooperation of large-T and telomerase. Similar observations have been made on hematopoietic progenitors (26). Telomerase alone is not sufficient (27) but is necessary to transform human primary cells. One explanation for the different efficiency of transformation of human and rodent cells such as REF may be that rodent cells have sufficient built-in telomerase activity and long telomeres.

3. Multistep oncogenesis in human diseases

Premalignancies (i.e. precursor lesions) are mostly asymptomatic lesions that become a cause for concern if untreated since they progress to malignant lesions after a variable period of time. There are several types of premalignant diseases. The main demonstration of multistep oncogenesis in humans came from studies on colorectal cancers, which often arise from premalignant polyps or adenomas (28). Early adenomatous polyps have mutations of the tumor suppressor APC. This protein functions in the WNT signalling pathway and localizes partly to the centrosome. Genomic instability is frequent in APC-mutated polyps (29). RAS mutations and other alterations occur later, in adenomas of larger size. Virus infection is a major cause of premalignant state. High-risk HPV16 leads to cervical carcinoma. HPV-infected cells express only two small viral early proteins, E6 and E7. Oncoproteins from high-risk strains interact with key regulators of the cell cycle; E7 inactivates RB and CDK inhibitors while E6 inactivates P53 (30,31).

Myeloproliferative disorders (MPDs) may be viewed as premalignancies at the chronic stage. MPDs are a clonal proliferative disease of hematopoietic progenitor cells with expansion and maturation of myeloid cells. Some MPDs are due to chromosomal translocations that result in the fusion of genes encoding tyrosine kinase with various partners. The BCR-ABL fusion protein is the oncogene responsible for chronic myeloid leukemia (CML). Other fusion proteins found in MPDs involve the JAK2 kinase, the PDGF receptors, or the FGFR1 kinase, including, for example a FOP-FGFR1 fusion. In a gene fusion, the partner gene provides a promoter that is expressed in hematopoietic progenitors, and a protein product with oligomerization motifs. It may also provide a specific addressing signal. We have recently shown that the FOP-FGFR1 protein is targeted to the centrosome in MPD cells. The oncogenic kinase induces substrate phosphorylation at the centrosome, sustained G1/S progression and cell proliferation (32-34). This is the first example of alteration of centrosome function without alteration in number in non-virally induced human cancers. At the chronic phase most MPDs do not show genomic instability and aneuploidy. The translocation is often the only obvious genome alteration, and MPD cells display structural alterations of the centrosome and of their genome mostly when secondary events occur, inducing a second phase called the acute phase. This fully malignant phase is characterized by the presence of blast cells that do not differentiate.

4. The control of the cell cycle

Stages of oncogenesis may be in tight relation with the cell cycle and its checkpoints. The cell cycle is divided in four phases, G1, S, G2 and M. Each phase is under the control of protein complexes that include positive and negative regulators. Cyclins and various kinases are primary components of cell cycle regulation (Fig. 1). Important duplication processes take place during the cell cycle, such as increase in size and protein content, centrosome duplication, DNA duplication, chromosome duplication and segregation. A ‘quality control’ is applied to each phase before the next phase begins, to detect dysfunctions and errors in these processes. Control is conducted by checkpoint complexes that stall the cell cycle until appropriate repair is completed (35). If no such repair is achieved the cell may pause or undergo senescence or apoptosis. Alteration of repair (controlled by caretakers), and checkpoint complexes (controlled by gatekeepers) is central to oncogenesis.

G1 checkpoints are not well defined but important processes such as increase in cell size and centrosome duplication are initiated in G1 and are thus controlled. The G1 phase can progress when the pocket proteins of the retinoblastoma (RB) family are phosphorylated by G1 cyclin-dependent kinases (CDK). Two families of CDK inhibitors (CDKN1 and CDKN2), maintain RB proteins underphosphorylated and block G1 progression. Regulators of G1 are very often targeted in human cancers (36,37); *cyclin D1, MYC*, and *CDK4* genes are recurrently amplified, while CDKN2A and PI3KCA, a subunit of the phosphatidyl inositol 3 kinase (PI3K), which regulates G1, are among the most frequently mutated proteins [COSMIC (Catalogue of Somatic Mutations in Cancer) database: http://www.sanger.ac.uk/genetics/CGP/cosmic/]. The G1/S checkpoint ensures that the DNA to be replicated is not damaged. The G2 checkpoint then verifies that DNA replication has been completed correctly. Finally, the mitotic checkpoint verifies that the spindle and chromosomes are correctly functioning to ensure equal DNA

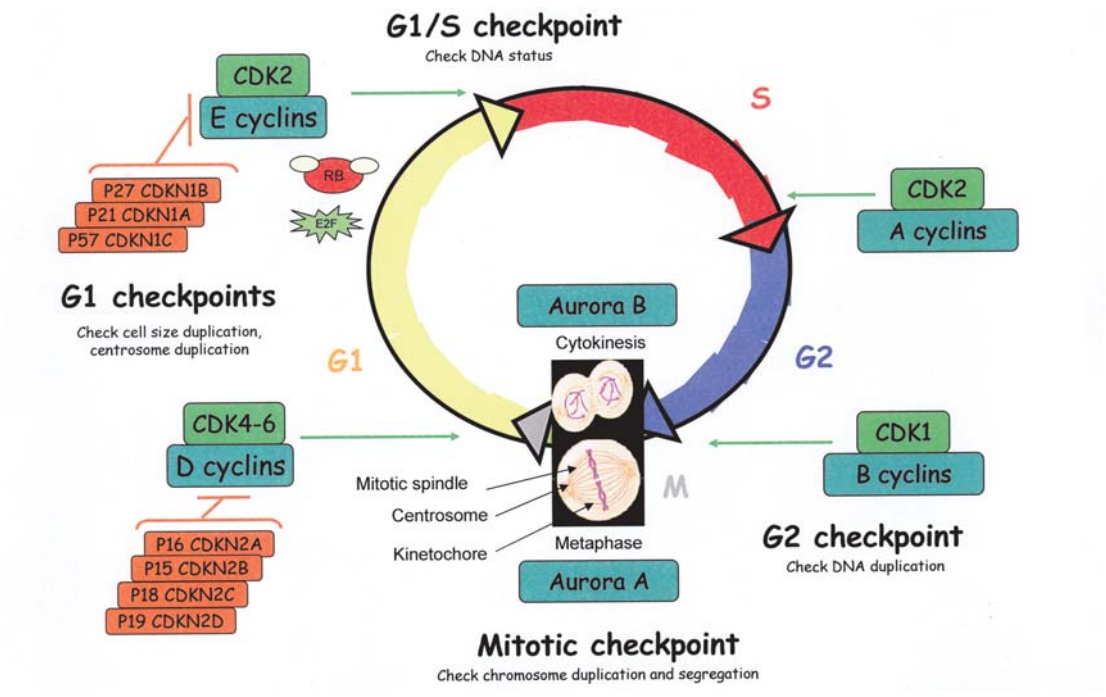

Figure 1. Representation of the cell cycle. The different phases of the cell cycle are represented with regulators of cycle progression and checkpoints.

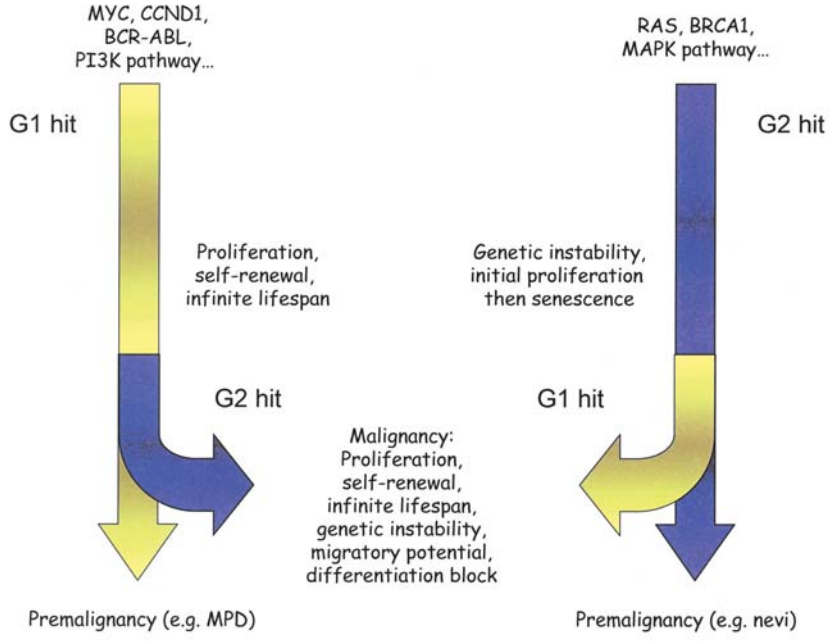

Figure 2. Two roads to cancer. In a first scenario, the first hit (oncogene amplification, constitutive activation of kinases, PI3 kinase pathway...) targets the G1 phase of the cell cycle and inactivates its intrinsic controls, principally RB and P53 controls. It leads to a premalignant stage characterized by a series of functional features, mainly self-renewal, immortality, and proliferation. The G1/S transition is facilitated, and the G1/S checkpoints are silenced; thus, mutations in DNA and centrosomal abnormalities can occur without the cell pausing or undergoing senescence or apoptosis. Some degree of aneuploidy may or may not be visible at this early stage. The second step occurs when either DNA or centrosome (or both) targeting provides the primed cell with another set of mechanisms allowing further release from intrinsic G2/M checkpoints and from extrinsic controls, as well as growth autonomy, and escape from (migration) and/or control (neo-angiogenesis) of the environment. In an alternate way, the G2 or M phases of the cell cycle are hit first by oncogene or tumor suppressor mutations and intrinsic checkpoints are altered. However, cellular senescence is activated as a stress-induced response. This scenario may be at work in some premalignant states (e.g. nevi). When G1 controls are secondarily affected a full malignant state is installed.

content distribution to the daughter cells. Several components of the G2 and M checkpoints (e.g. P53, BRCA1, BRCA2, ATM, ATR, MDM2, Aurora A) are altered in human cancers (35).

5. Cooperation in cycle

We hypothesize that two main oncogenic hits (each hit being the result of one to several genetic events) cooperate to

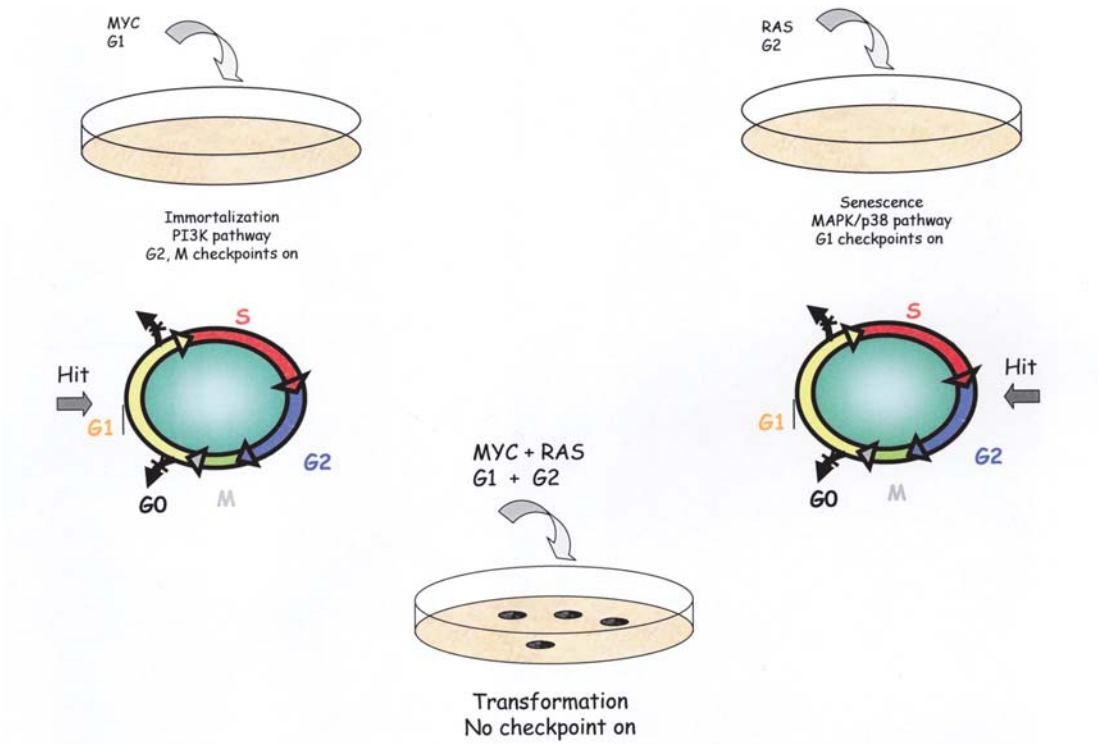

Figure 3. Oncogene cooperation revisited. Cooperation of oncogenes in the transformation of cultured primary cells can be seen as a sequence of G1 alteration (immortalization by tempering with the division of a stem cell) and G2/M alteration (induction of senescence rescued by G1 checkpoint relief).

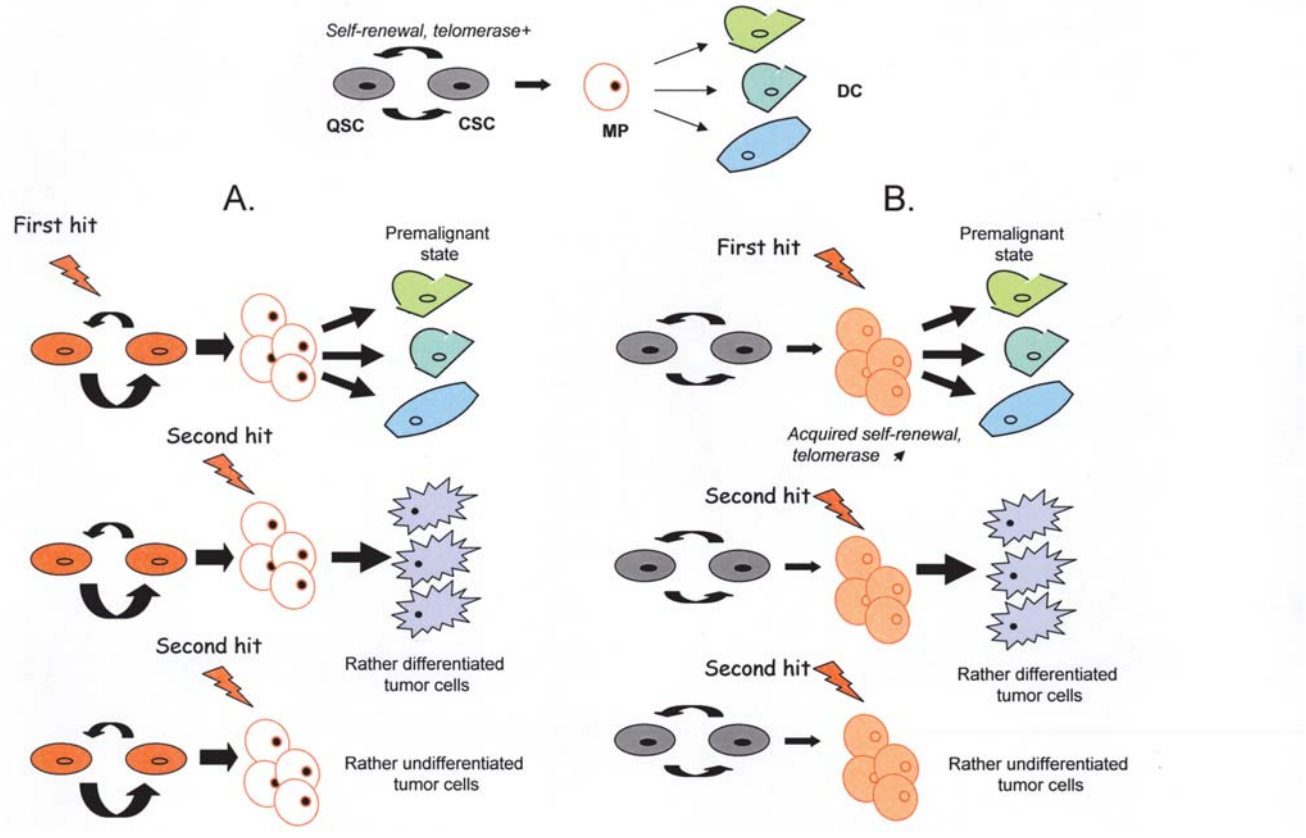

Figure 4. Two scenarios for the epithelial targeted cell. A schematic representation of a cell maturation pathway is represented at the top. The number of stem cells present in a tissue is regulated according to needs and controlled by asymmetric division. Via asymmetric division a dividing pluripotent stem cell gives birth to stem cell (self-renewal) and to a proliferating progenitor cell (multipotent progenitor, MP), which is engaged in differentiation but still able to give rise to different types of differentiated cells (DC). A, in a first scenario, stem cells are targeted and proliferate, but differentiation and diversity are preserved normally or almost normally. After a second hit (single or series of additional mutations), which occurs either in an altered daughter cell or in a primed stem cell itself, differentiation is altered, severely or not. B, in a second scenario, the first hit occurs in proliferating MP (also called transient-amplifying cell) that acquires self-renewal and infinite lifespan.

transform cells to a malignant phenotype. There may be two ways to initiate a malignant disease. The first oncogenic hit may target either the G1 phase or the late phases of the cell cycle (Fig. 2). The second oncogenic hit reciprocally targets either S/G2/M or G1.

A G1>G2 sequence. The first hit modifies the G1 phase of the cell cycle by targeting the centrosome or by another pathway. Mutations can directly target gatekeepers such as RB (6,38). Amplification of *MYC* and *cyclin D1* genes, and overexpression of the corresponding proteins, target the G1 phase. Alterations of the PI3K pathway seem also a primary determinant of G1 activation. The result is sustained G1/S transition and proliferation. This first series of acquired functional alterations results in escape from senescence and quiescence, and provides unlimited survival (i.e. immortality) and proliferative capacity. If this event occurs in a stem cell endowed with self-renewal, extended lifespan and high telomerase activity, then a premalignant state is established. The altered cells are forced to proliferate but they are initially genetically and functionally normal, their G2/M checkpoints are not activated, and their progeny can still respond to differentiation signals. At this stage, the oncogenic process begins to create conditions for aneuploidy and genetic instability. Without a forced G1/S passage a cell with mutations, chromosome abnormalities and/or centrosomal dysfunction will undergo cell cycle arrest upon activation of the checkpoints; abnormalities will be repaired or the cell will undergo apoptosis. Sustained G1/S transition allows the premalignant cells to continue cycling even if a certain number of centrosome abnormalities, chromosome missegregation or DNA replication errors occur and eventually accumulate. At this stage, centrosome abnormalities and chromosomal instability may be detected (39).

G2/M blocks will arrest most cycling cells but inexorably a second event will overcome the checkpoints. The second event stems from the random accumulation of errors in the DNA and/or centrosome of the continuously cycling cell. It provides the proliferating clone with a more aggressive armament, which includes growth factor and stroma independence, absence of response to differentiation signals and absence of polarity. Initially, sustained but environment-dependent growth induces unbearable constraints on the altered cells as their number increases. Independence from (cell-cell communication) and adaptation to (neoangiogenesis) the environment are major steps in malignant progression. Mutation of RAS small GTPases and activation of the MAP kinase (MAPK) pathway may be one way to trigger signaling pathways necessary for growth autonomy. The second hit often relies on alterations of centrosomes and release from G2/M and mitotic checkpoints (e.g. P53 mutations) to create chromosomic instability and aneuploidy.

The second oncogenic hit results from additional changes in genotype and increased genomic instability, and is the hallmark of fully malignant cells. In contrast to the fist hit, the number of aggressive features depends on the type and number of genomic alterations and may be variably present in the cancer cells. The consequences of genome instability create a repertoire of various alterations and the result is a variety of genotypes and heterogeneous cell behavior. The second hit thus drives tumor heterogeneity, which in turn leads to heterogeneity in disease prognosis and evolution. G2/M checkpoints may be directly affected by mutations in control proteins such as Aurora A, BRCA1, BUB1, CHEK2, MAD2, or P53 (40). However, the second hit does not necessarily generate genomic instability. Approximately 50% of human acute leukemias have a normal karyotype. The major manifestation of oncogenesis is here a block of differentiation at the immature (blast) cell stage. This block may be one of the possible methods by which proliferating cells escape environmental control.

A G2>G1 sequence. The late phases (S to M) of the cell cycle can be hit first by an oncogenic offense. RAS oncogenes may affect the M phase (41). Expression of a mutated RAS in mouse skin epidermis induces papillomas (42) and in a transgenic mouse model induces lung adenomas (43). However, in this case, premalignant cells show signs of senescence. RAS oncogenes induce premature senescence through MAPK/P38 activation (44) and activation of a set of tumor suppressors including RB, P16 and P53. Transgenic mice overexpressing cyclin A (which acts in the S/G2 phases of the cell cycle) in the mammary gland develop nuclear abnormalities and increased apoptosis (45). Tumors progress to carcinomas only in variants in which secondary events overcome senescence by relieving a G1 block. This suggests the existence of a G1 checkpoint that arrests cells with oncogene-induced defects in S/G2/M phases (e.g. DNA damage, replicative stress) and leads to premature senescence. The order of events is important in colon cancer; KRAS mutations do not progress to colon cancer without prior APC alteration (46). Expression of BRAF, a downstream mediator of RAS, in nevi (benign melanocytic tumors) induces proliferation but also senescence; the arrest is overcome by SV40 large-T antigen, which inactivates both RB and P53 (47,48). A number of benign mesenchymal tumors show dysregulation of the HMG2A protein, a non-histone component of chromatin (49). Targets of HMG2A are *cyclin A* and DNA-repair gene *ERCC1*. HMGA proteins play a role in stabilization of senescence (50). Co-amplification and overexpression of *HMGA2* and *CDK4* genes at chromosomal region 12q14 occurs in some human cancers such as breast cancers and glioblastomas, and may cooperate to transform cells. HMGA2 is also targeted in cases of myelodysplasia (51). Myelodysplasia is a pathological syndrome that often precedes acute leukemia. It is characterized by bone marrow failure, multilineage dysplasia and abnormal differentiation, and peripheral cytopenias (52). It is possible that myelodysplasia is caused by oncogenetic events inducing some level of senescence. Subsequent G1 targeting may transform myelodysplasia to acute leukemia.

It is thus possible to re-interpret oncogene cooperation in the context of cell cycle activation (Fig. 3). The order of cell cycle events may be variable but the same steps must be targeted. Checkpoints should be removed and cycling should be clear of brake signals at any phase. The order of events may be random or may depend on specific cell permissivity to certain oncogenic alterations (themselves resulting from susceptibility to mutations or amplifications for instance). Whatever the premalignant state, the first hit will eventually create the conditions (forced cycling for G1, instability for

G2/M) for the second hit to happen. In this scheme, one level of complexity is determined by the role of cancer genes; although we can try to assign oncogenes and tumor suppressors to G1 or G2/M, many cancer genes (e.g. P53, RAS or PI3 kinase) may act at several phases of the cell cycle, perhaps depending on the cell or environmental context.

6. Four potential ways to cancer

Several models have been proposed to describe the origin of cancer cells (53-55) (Fig. 4). In the 'cancer stem cell model', a cancer cell derives from a stem cell that has suffered an oncogenic hit (Fig. 4A). Quiescence, self-renewal, asymmetric division, prolonged lifespan and multipotency are intrinsic features of stem cells. In regulated periods of tissue maintenance, a stem cell undergoes asymmetric division, i.e. it divides into one copy of itself and a daughter cell that will enter a differentiation pathway. Stem cells in some renewing tissues undergo more than 1000 divisions in a lifetime with no morphological sign of senescence (56). The first oncogenic event(s) should thus only trigger sustained asymmetric cell division and prevent the stem cell from entering quiescence. This will lead to unregulated expansion of stem cells and progenitors. Alternatively (Fig. 4B), a cancer cell may derive from a more mature proliferating progenitor cell that has acquired stem cell properties, i.e. self-renewal and immortality. In this case, the fist oncogenic event(s) enable(s) the cell to self-renew. The gene expression programs associated with acquisition of self-renewal have begun to be deciphered (57). It is not known which model if any predominates in human tumors. The fact that three genes were necessary to transform human primary cells in culture (26) may mean that the normal primary cells used in culture for the experiments did not contain enough stem cells and that cells on their way to differentiation but not stem cells were targeted. If the stem cell model is true, transformation of isolated stem cells should not require TERT. Immortalization of $CD34^+$ stem/progenitor cells of human cord blood is achieved by human papillomavirus type 16 E6 and E7 oncogenes (27). However, TERT is needed for genome stability.

In both models, depending on the type of genome alterations and on the environment, the cells from the tumor mass may progress along a normal differentiation pathway (e.g. luminal-like breast cancers with hormone receptors) or remain blocked at an immature stage. It is likely that the two models are found in human tumors. Depending on their functions, some oncogenes, such as MLL gene fusions, can confer properties of stem cells to progenitors bound to undergo differentiation or apoptotic cell death, while others target only self-renewing stem cells (58). A possibility is that some events, such as perhaps the oncogenic targeting of the centrosome, trigger in the same time proliferation, survival and self-renewal and can target indifferently stem cells or committed progenitors. Whatever the targeted cell, asymmetric division is likely to be a key altered process (59,60). Proteins of the WNT pathway, such as GSK3, APC or ß-catenin, or of the NOTCH pathway, may intervene in this process.

Thus, to summarize, depending on the targeted phase of the cell cycle (G1 or S/G2/M) and the targeted cell (stem cell or progenitor), there could be four theoretical ways for a first hit to initiate a cancer. The second hit provides the missing components of the necessary oncogenic spectrum (7,8). However, some of these four ways may not always be possible or efficient, depending on the type of genetic alteration. For example, too much tempering with the self-renewal program may prevent an oncogene from efficiently targeting a stem cell. A possibility could be that stem cells are targeted in G1 but cannot initiate a tumor if one of the late cell cycle phases has been targeted, whereas progenitor cells, already cycling, might be more prone to G2/M attacks to acquire self-renewal.

Our sequential cell cycle model may fit the oncogenic model of some neoplasias but does not aim at explaining all of them. Oncogenic hits may not be related to cell cycle targeting. For example, self-renewal and block of differentiation may be acquired by other means (57). Nevertheless, we believe that in many tumors the G1 phase is a frequent primary target. Most, if not all, human cancers show a deregulated control of G1 progression (36,61). In terms of molecular pathways, oncogenesis major targets are the RB and P53 pathways, as already proposed (8,38). These convergent pathways contain several targets (RB, P53, cyclins, CDKs, P16/CDK2NA) often altered in human tumors (36). Similarly, genetic instability and aneuploidy, common features of tumors, are frequent consequences of G2/M alterations.

7. Further questions and perspectives

After the initial phase that followed the discovery of the first oncogenes, which was full of enthusiasm and optimism, cancer research was dominated by the view that things may not be as simple as they appeared to be. Then, after having decrypted some of this complexity, we now consider things may actually be simpler than we thought. We may have identified the targeted cells and genes. Cells from different types of cancer may thus share not only common altered pathways but also common stem cell markers. However, even if the main culprits and some general rules are identified, many questions need to be answered:

For each cancer type, when and where do the different oncogenic hits occur? Do they target a particular component of the cell such as the centrosome? Do they target stem cells, progenitors or both? Is it important for prognosis to know the sequence of events? For example, will tumors initiated by a G1 hit fare better or worse than tumors initiated in G2/M?

What determines specificity? Why are some of these ubiquitously-expressed targets more specific to some tumor types? For example, HRAS, KRAS, NRAS or BRAF mutations occur frequently in some types of cancer but are rare in others. A possible explanation for oncogene specificity of human cancers is that specificity may be partly driven by the order of events. For example, tumors with RAS mutations could be the ones initiated by a first hit in G2/M. Thus, a combination of three factors could determine specificity: the type of genetic alteration, the type of targeted cells, and the order of events in the cell cycle.

What determines systematic progression? What distinguishes benign non-precursor lesions from premalignant tumors? A role for inflammation in cancer progression has

been suggested by some recent studies (62,63). The role of the stroma and other factors is also extremely important (64). Should we search to distinguish premalignant syndromes with genomic instability from those without? It is possible that the clinical courses of the two types differ. The research in this domain is still in its infancy. It seems that much could be learnt by studying models of these diseases. More research on premalignancy syndromes is certainly needed. In this context, the distinction between 'G1 premalignancies' and 'G2 premalignancies' could be important.

Our hypothesis may help in designing experimental strategies. The identification of a G1 alteration should lead to a search for secondary events in G2/M and vice versa. Breeding different mouse models of cancer could also be based on G1/G2 cooperation. The hypothesis may also help in designing therapeutical strategies. The use of a combination of drugs that target both G1 and G2 may be more efficient at compromising the growth of a tumor than a combination of drugs specific of one phase only.

Acknowledgements

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