1. THE ORIGIN OF CANCER

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INTRODUCTION

Cancer is a complex genetic disease. Work over the past fifty years confirms that the genetic alterations found associated with human cancers impair the function of pathways critical to controlling cell growth and differentiation. In aggregate, these genetic mutations allow a malignant cell to acquire a set of biologic attributes leading to autonomous proliferation and metastatic spread. Despite this paradigm, the precise nature and timing of each of the events that conspire to program the malignant cell remain incompletely understood.

Although familial cancer syndromes are responsible for only a minority of human cancers, the study of these kindreds has facilitated our understanding of cancer genetics. In many such syndromes, individuals inherit one defective, predisposing allele in the germline, and only later in life do they acquire a second loss of function mutation. As first described by Knudson, this "two hit" hypothesis helps explain such inherited cancer syndromes such as retinoblastoma and Wilms' tumors (1). Although the tumors in these patients express mutations in specific inherited genes, the finding that these tumors also harbor a myriad of other genetic changes indicates that further alteration by somatic mutation are required for tumor development (2).

However, the majority of human cancers lack a readily definable predisposing genetic defect and appear to be the result of a concert of acquired genetic alterations. Work from many laboratories, using both patient-derived material and experimental cancer models, have begun to define these malignant genetic mechanisms.

In spontaneously arising human cancers, we still cannot determine the exact number and nature of genetic alterations involved in the process of transformation from a normal cell to a malignant one. Since cancer encompasses more than 100 different types of malignant diseases with great heterogeneity of clinical characteristics, every tumor could hypothetically be completely unique. Thus, cancers, in general, could harbor an undecipherable number of genetic and epigenetic changes leading to their development.

Alternatively, pathogenesis of human cancers may be dependent on a distinct set of genetic and biochemical alterations that apply uniformly to most if not all human tumors. These changes may alter the functions of specific pathways involved in important biological functions and facilitate malignant transformation, endowing cells with specific changes in cell physiology, termed "acquired capabilities," ensuring their survival and continued success (3). In particular, cancer cells generate their own mitogenic signals, proliferate without limits, resist cell cycle arrest, evade apoptosis, induce angiogenesis, and eventually devise mechanisms for invasion and metastasis.

GENETIC REQUIREMENTS FOR CANCER

Epidemiologic analyses have shown that four to six rate-limiting events must occur before a tumor becomes clinically apparent (4,5). The changes that must occur are genetic and/or epigenetic in nature. Most of these events result from somatic mutations that occur infrequently or are induced by carcinogen exposure, and only in aggregate do they lead to the tumorigenic state.

The colorectal carcinoma model

In a seminal series of studies, Vogelstein and his colleagues described a stepwise genetic history of colorectal tumors (6). Since colorectal carcinoma develops intraluminally and tissue is readily available for examination, specific histopathological alterations that occur in cancer development are readily observed in different stages. By studying tissue derived from specific histopathologic stages, ranging from normal colonic epithelium to frank carcinoma, they catalogued genetic alterations specific for each stage, thereby developing a model that dissected an accumulation of separate genetic mutations that could in combination lead to malignancy (7,8).

A vast majority of early adenomatous polyps were found to exhibit an inactivated mutant form of the tumor suppressor gene, adenomatous polyposis coli *(APC)* (9). Alterations in this gene had been previously shown to be responsible for Familial Adenomatous Polyposis (FAP) (10,11). However, patients with germline mutations of *APC* have a greater risk for but do not necessarily develop colorectal cancer. In addition to the germline mutation, somatic mutation of the wild-type *APC* allele must also occur (9,12).

When they investigated intermediate size adenomas, they found that approximately half carry activating mutant *RAS* oncogenes (6,13). Interestingly, normal colonic epithelium harboring *RAS* mutations alone, do not lead to neoplasia (14), and these cells may eventually succumb to apoptosis (15), suggesting that other genetic alterations are necessary for *RAS* mutation to contribute to tumor formation. In a subset of larger

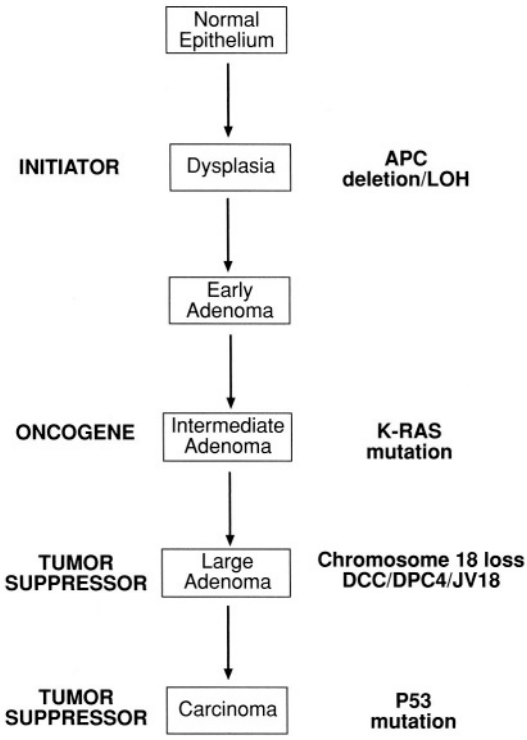

Figure 1. Genetic Model of Colorectal Carcinoma Development. Multiple genetic alterations are found at different stages of development from pre-malignant lesions on to frank carcinoma. These genetic lesions may represent necessary alterations to progress to the next developmental phase toward cancer.

adenomas, alteration of a chromosome 18-associated tumor suppressor gene such as *DCC, DPC4,* or *JV18,* were common. Finally, 80% of colorectal carcinomas show evidence of genetic alterations of the *P53* tumor suppressor gene (16). Surprisingly, however, patients with Li-Fraumeni syndrome, who have germline mutations of *P53*, do not have a higher risk of colorectal cancer development and do not even tend to develop polyposis (17). Thus, although both *P53* and *RAS* play individual roles in colon cancer pathogenesis, these observations suggest that oncogenesis cannot be accomplished by a random accumulation of mutations. The order of alteration, as well as the necessity for an initiator like *APC* deletion, may both be important determinants for formation of the resultant tumor (Figure 1).

These observations provide evidence that the history of human cancer follows a stepwise progression of genetic events. This model, however, demonstrates only one of many potential pathways to the neoplastic state.

While these observations in colorectal cancer certainly suggest that all cancers progress through a similar series of ordered events, no other human cancer has been similarly mapped, and abundant evidence indicates that specific mutations differ among

particular cancers. Understanding the combination of events required in each type of human cancer remains an important goal of future studies.

EXPERIMENTAL MODELS

Initial studies of human cancer cells were limited to samples obtained from tumor biopsy specimens. To facilitate further study, cells from these tumors were frequently adapted into cell lines that grow in culture (18). These cell lines are useful for many purposes, however, it is impossible to determine the order or even a set of defined genetic or biochemical changes that lead to neoplastic development. Complicating matters further is the high likelihood that additional genetic alterations are acquired over time through propagation in culture.

Recently, transcriptional profiling has been helpful in evaluating the simultaneous expression of thousands of genes in particular cancers or cancer cell lines (19,20). Unfortunately, while these studies have provided us with tools to better classify cancers, they have not yet yielded insight into the functionally important gene expression changes required for cancer growth. It is still impossible from these analyses to determine which genes have true functional roles in the transformation to the malignant state. Thus, a complementary approach to studying the genetic alterations necessary to form a tumor is to transform normal cells, *in vitro,* by serially introducing multiple oncogenes. An alternative method of cancer modeling is through the production of genetically altered mice harboring specific alterations associated with human cancer.

Rodent cell transformation

In rodent systems, single oncogenes fail to transform primary cells without the presence of prior predisposing mutations (21,22). In contrast, two introduced oncogenes convert embryonic rodent cells to a tumorigenic phenotype (23,24). These observations indicated that the conversion of normal cells into cancer cells requires multiple genetic changes to occur.

Collaborating oncogenes that induced transformation in these cultured rodent primary cells included *Myc/Ras* or *E1a/ ras* (23,24). Further confirmation of this collaboration through transgenic mouse experiments occurred when a *Ras* or a *Myc* transgene was placed under the control of mammary- or prostate- specific promoters (25,26). Dysplasia in promoter specific organs developed at high rates in the transgenic mice expressing single oncogenes, but frank tumors did not develop unless mice expressed both transgenes. These findings support the concept that specific oncogenes collaborate to aid in tumor development *in vivo,* as well as in cultured cells.

Barriers to human cellular immortalization

While two oncogenes appeared to suffice to transform rodent cells, the transformation of primary human cell lines proved to be more complex. This difference is in part because human cells require more genetic alteration to bypass the barriers of immortalization (Figure 2). When normal human cells are grown in culture, their proliferative potential is limited and they eventually enter an irreversible, quiescent state, termed mortality stage 1 (M1) or replicative senescence (27). Although these cells are still viable, they can no longer be stimulated to divide. The exact trigger for entry into replicative

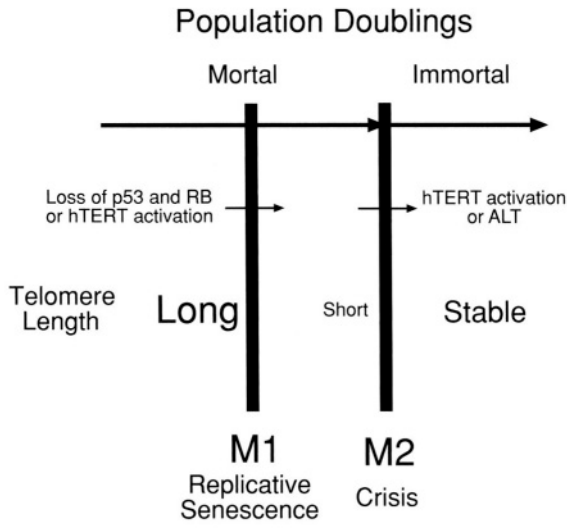

Figure 2. Barriers to Human Cellular Immortalization. Normal passage of cells is halted at MI unless this barrier is bypassed by p53 and RB inactivation or hTERT expression. These cells can then continue dividing until their telomeres become critically short at M2. hTERT expression or ALT allows telomere length stabilization and cellular immortalization.

senescence is still unclear, although there are a variety of stimuli that have a role in this process (28).

Pre-senescent cells can be experimentally manipulated to bypass replicative senescence through ectopic expression of certain genes. Expression of *hTERT,* the catalytic subunit of telomerase is capable of bestowing some but not all primary cells with immortality (29,30). Another mechanism of bypassing this first proliferative barrier is through simultaneous abrogation of the *P53* tumor suppressor and retinoblastoma *(RB)* pathways (28). Expression of viral oncoproteins, such as SV40 large T antigen (31) or human papillomavirus E6 and E7 oncoproteins (32), which bind to and inactivate p53 and RB (33), respectively, offer experimental methods of achieving this dual inactivation.

Cells that lack telomerase overexpression but that express the above mentioned viral oncoproteins, may then undergo another 10–20 population doublings before they encounter mortality stage 2 (M2) or crisis. Here the vast majority of cells have short telomeres (34), display karyotypic abnormalities (35), and die by apoptosis (36). Since in culture telomeres shorten by 50-100 base pairs during each cell replication (37), ongoing passage allows telomeres to shorten to a critical length. This results in an inability to protect the ends of chromosomes, leading to genomic instability and triggering crisis (38).

Rare variants, approximately 1 in 10 million cells, emerge from crisis, and have infinite proliferative capability (31). These cells typically exhibit stable telomere lengths and express the *hTERT* gene with preserved activity (38), which is felt to be expressed

at low levels in normal cells (39). These findings have been corroborated by observations in post-senescent, pre-crisis cells that avoid crisis and proliferate indefinitely after transduction with *hTERT* (40–42). However, a subgroup of cells may become immortal without significant *hTERT* or telomerase expression (43,44). These cells have a separate mechanism of telomere length maintenance, termed alternative lengthening of telomeres (ALT), which likely involves recombination (45).

Human cell transformation

The observations that suggested that human cell immortalization is more complex than rodent cell immortalization also complicated attempts for experimental transformation of human cells. From this set of observations, Sager and her colleagues postulated that the senescence program is a barrier to cancer development (46,47). Recent work, however, has begun to identify combinations of genetic alterations that suffice to confer human cellular transformation.

Thus, specifically targeting each of the barriers of immortalization by introduction of the SV40 Early Region, which encodes the large T oncoprotein, in combination with the *hTERT* gene into normal human fibroblasts and kidney cells suffice for immortalization (48,49). Since the SV40 Early Region also encodes for small t oncoprotein, subsequent transduction with oncogenic *RAS* results in the ability to develop tumors in immunocompromised mice, hence transformation. Additional studies have revealed this combination of genetic alterations to be sufficient to transform multiple cell types, including cells of mammary (50), lung (51), prostate, ovarian, mesothelial (52), endothelial, and neuroectodermal (53) origin. Thus, it is necessary to understand the roles of these basic genetic elements involved in transformation in regards to the critical pathways that they effect. For example, the large T oncoprotein may functionally inactivate the p53 and RB pathways, but the inactivation of these two pathways may in sum not equal the effects of the oncoprotein alone, as there may be additional functions gained with large T. Thus, a myriad of other genetic mutations that lead down similar or parallel paths may also bestow specific "acquired capabilities," leading to similar functional endpoints or the neoplastic phenotype.

MOLECULAR CHANGES

Experimental evidence has allowed the delineation of a few crucial pathways in human primary cell transformation. Although there are many important cellular capabilities, allowing a normal cell to bypass cell cycle arrest checkpoints, escape apoptosis, guard against crisis, and provide its own mitogenic signals, may be sufficient to allow for transformation to the oncogenic phenotype. These basic genetic elements may be generalized to most human cancers, however, specific alterations that contribute to oncogenesis are found in some cancers, such as thyroid cancer. These well-defined specific molecular alterations involved both in thyroid-specific and general malignant transformation are described below.

The *P53* tumor suppressor gene

Perhaps one of the most common alterations in human cancers is mutation of the *P53* pathway, found altered in most, if not all, human cancers (54). Loss of wild-type

p53 protein expression, in conjunction with gain-of-function from mutant proteins (55), contribute to acquisition of specialized cell properties, such as proliferative and survival advantages. p53 performs these tasks by acting as a transcription factor induced in response to DNA damage, hypoxia, or oncogene activation (54,56). This, in turn, initiates a program of gene regulation leading down at least two major separate pathways, one for cell cycle arrest to allow time to repair damaged DNA and another for apoptosis to trigger the cell to euthanize (54,57).

Wild-type p53 protein may act as a cellular defense mechanism through its effects on cell cycle arrest and apoptosis, both major obstacles to tumor formation. Cells that are unable to arrest and correct DNA damage have increased potential to develop genetic instability with ongoing replication. At the same time, survival of a neoplastic cell, also includes evasion of apoptosis, preventing the cell-suicide program from taking an antitumor effect. Thus, abrogation of wild-type p53 function, may be sufficient in some tumor types to dismantle the apoptotic machinery (58). However, in other tumors, specific components of the apoptotic cascade, such as bcl-2 (59), Akt (60), or caspases (61), must also be inactivated.

p53 regulates a number of genes involved in the cell cycle. One of these proteins, $p21^{CIP1}$, is upregulated by p53 and inhibits the cyclin dependent kinases, resulting in G1 cell cycle checkpoint arrest. Another is Hdm2, a negative regulator of p53, which is also positively regulated by p53 protein itself (Figure 3). Hdm2 physically binds p53

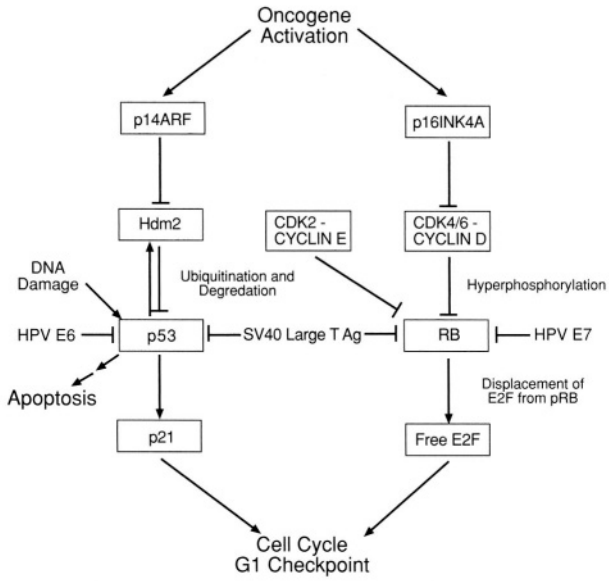

Figure 3. The P53 and RB tumor suppressor pathways. These are both central molecular pathways that are often dysregulated in cancer. Each of these tumor suppressors are regulated by multiple proteins, and disruption can occur at any of these points in human cancer. The role of p53 in apoptosis entails a complex pathway that is not shown on this diagram. Arrows signify activation of the target while blunt lines act in an inhibitory fashion.

protein, inhibiting its activity as a transcriptional factor, meanwhile catalyzing p53 ubiquitination which marks it for proteasomal degredation (62) Hdm2 is itselfregulated by $P14^{ARF}$, another tumor suppressor whose protein product binds to and inactivates Hdm2 (63).

While *P53* may be directly mutated in over half of all human cancers, in some tumors no *P53* mutation is observed, yet other genes in the pathway are altered. For example, Hdm2 can be overexpressed and antagonize p53 protein function in a variety of cancers, including B-cell lymphomas (64), melanomas (65), and breast cancers (66). Other tumors harbor $P14^{ARF}$ deletions or suppression by methylation, permitting Hdm2 to remain active and drive the degredation of p53 (63,67). Thus, a various array of genetic and biochemical alterations can converge to enforce a common resultant phenotype, aiding in tumor development and progression.

As will be described in greater detail elsewhere, in thyroid carcinoma, *P53* alterations have been found more frequently in both poorly differentiated and undifferentiated thyroid carcinomas (68). Thus, p53 may have a role in the dedifferentiation process. A combination of mutation (69), loss of heterozygosity (70), and overexpression (71,72), presumably from decreased degredation, have all been found in thyroid cancer, again declaring the importance of this critical pathway. (See Chapter 8).

The retinoblastoma (RB) protein

Regulation of passage through the G_1 checkpoint of the cell cycle is one of the most important roles of the retinoblastoma protein (73). In its hypophosphorylated form, this protein inhibits cellular commitment to mitosis by blocking cell cycle entry into S-phase. In that state, it is bound to various members of the E2F family of proteins (74). These RB-E2F complexes can inhibit gene transcription by multiple methods: (1) Interfering the ability of free E2Fs' to act as transcriptional factors for cyclin E, cyclin A, and multiple other genes necessary for DNA replication (75) (2) Actively recruiting histone deacetylases (HDACs) (76) and other chromatin remodeling factors to E2F responsive promoters (77).

RB inactivation is a crucial step in allowing a cell to pass the G_1 checkpoint and continue through the cell cycle (Figure 3). Normally, one of the cyclin D subtypes (D1, D2, or D3) assembles with one of the cyclin-dependent kinases, CDK4 or CDK6, and cyclin E binds to CDK2. These active holoenzymes phosphorylate RB proteins. Once in a hyperphosphorylated state, RB is unable to bind E2F or HDACs, and releases the repression on genes required for S-phase entry.

Several other tumor suppressor genes also contribute to the phosphorylation status of pRB. For instance, $p16^{INK4A}$ inhibits the activity of cyclin D-dependent kinases to prevent RB phosphorylation and halt cell division (78). The cyclin E-CDK2 complex is inhibited by both $p21^{CIP1}$ (79) and $p27^{KIP1}$ (80). However, when a strong mitogenic stimulus is present, increased cyclin D1 tends to complex with CDK4, and this combination sequesters $p27^{KIP1}$. This leaves cyclin E-CDK2 free from $p27^{KIP1}$ inhibition to phosphorylate and inactivate RB. E2F, as a result, dissociates from hyperphosphorylated RB and acts as a transcription factor for a number of responder genes,

including cyclin E. The transcription of these responder genes are required for cell cycle progression through the G1 restriction checkpoint, facilitating cellular division.

Like *P53,* mutations in *RB* or its associated tumor suppressor genes occur frequently, and disabling this pathway may be required for the formation of human cancer cells (81,82). For example, loss of function mutations of *RB* also can be found in osteosarcomas and lung cancers, particularly small cell tumors (81). Although *RB* mutations do occur in non-small cell lung carcinomas, they appear to be present in approximately 20–30% of cases as compared to 80% of the small cell subtype (75). However, $p16^{INK4A}$ loss is evident in over half of all non-small cell lung cancers. Inactivation of $P16^{INK4A}$, by genetic lesions or by methylation, disrupts the RB pathway in a large array of other cancers, including pancreatic, breast, glioblastoma multiforme, and T cell ALL (67,75). Cyclin D1 overexpression drives the cell cycle forward and can also substitute for RB inactivation, as noted in breast cancers (83) and mantle cell lymphomas, where there is juxtaposition of the cyclin D1 gene with the immunoglobulin heavy chain promoter enhancer via a t(11:14) translocation (75). Cyclin E overexpression in breast cancers have also been noted and may help drive past the *RB* inhibition checkpoint in G1 (84). Finally, in many cervical cancers, human papillomaviruses (HPV) E7 oncoprotein sequesters and tags RB for degredation (85). Even in those cervical carcinomas that do not express HPV E7, *RB* somatic mutation is detectable. Alterations in the *RB* pathway seem to be mutually exclusive, as usually only one component of the pathway is mutated or lost; nonetheless, convergence on the loss of growth suppression by RB does seem to exist in the majority of human cancers (81).

However, the role of the *RB* in human thyroid cancer remains unclear. Although there are several human immunohistochemical studies (86–88) that remain inconclusive as well as studies evaluating *E2f* and *Rb* in rodents (89–91), definitive molecular evidence for the role of *RB* in human thyroid cancers is lacking. (See Chapter 8).

Mitogenic stimuli and oncogenic *RAS*

Normal and cancer cells differ in their innate ability to proliferate in the absence of mitogenic stimulation. The presence of surrounding growth factors are crucial for the continued proliferation of normal human cells. Cancer cells, in contrast, have reduced their dependence on external stimuli due to the activation of oncogenic mutations that generate constitutively active mitogenic signals (92). For example, alterations in growth-factor receptors, such as *HER2/NEU* amplification in breast cancer (93,94) or epidermal growth factor receptor mutation in most carcinomas (95), function as autonomous growth stimuli.

In human thyroid cancer, multiple activating receptors have been implicated in disease pathogenesis. Characteristic chromosomal rearrangements linking the promoter and amino-terminus domains of unrelated gene(s) to the carboxy-terminus of the *RET* gene result in a constitutively active chimeric receptor, termed (RET/PTC). This event may initiate papillary thyroid cancers (96). Constitutive activation of this mutant kinase promotes interaction with SHC adaptor proteins, intermediates in the *RAS* signaling pathway (97). Although rare, another early event in papillary thyroid cancers, may involve rearrangements of specific TRK tyrosine kinase receptors (98).

Both epidermal growth factor receptor (EGFR) and its ligands, epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α), are also widely expressed in both normal thyroid and thyroid neoplastic tissue (99,100); however, EGF has a higher binding affinity for neoplastic thyroid tissue when compared to normal tissue (101). EGF and its receptor stimulate proliferation of thyroid cancer cells and enhance invasion (102), suggesting their potential role in malignant progression.

Multiple intracellular protein networks exist downstream of growth factor receptors that can become constitutively active in a mutated state, conferring a growth-inducing effect. As discussed above, introduction of one of these aberrant signals, *H-RAS,* turns an activating switch on and facilitates malignant transformation to previously immortalized human and rodent primary cells. (See Chapter 7).

Various RAS proteins, members of a large superfamily of low-molecular-weight GTP-binding proteins, control several crucial signaling pathways that regulate cell proliferation. Their ability to effect downstream intracellular signaling proteins first rely on post-translational farnesylation to localize the *RAS* protein to the cell membrane. Then the ratio of biologically active RAS-GTP to inactive RAS-GDP depends upon the presence and activity of various guanine nucleotide exchange factors (GEFs) and their antagonists, GTPase activating proteins (GAPs) (103).

Multiple effector pathways lay immediately downstream of *RAS* (Figure 4). The RAF family of proteins, which can trigger a cascade of phosphorylating events through the mitogen-activated protein kinase (MAPK) pathway, leads to cell cycle progression. There is resultant ERK-mediated transcriptional upregulation of angiogenic factors, and increased capability for invasiveness through expression of matrix metalloproteinases. Through RAS stimulation of phosphatidylinositol 3-kinases (PI3Ks), RAC, which is a Rho family protein, can also increase invasiveness through its effects on the actin cytoskeleton. PI3K also triggers a strong anti-apoptotic survival signal through Akt/protein kinase B (PKB). Much like Akt, RALGDS, which is activated by RAS, inhibits the Forkhead transcription factors of the FoxO family which have a role in cell cycle arrest through induction of $p27^{KIP1}$ and apoptosis through the expression of BIM and FAS ligand (104). Finally, phospholipase C (PLC) is another RAS effector which promotes activation of protein kinase C and calcium mobilization (105). Alterations in the RAS proteins or their downstream effectors can therefore have the potential to lead to constitutively active signals, aiding the oncogenic phenotype. (See Chapter 7).

Activating point mutations of *RAS* occur in approximately 20% of human tumors, most frequently in pancreatic, thyroid, colorectal, and lung carcinomas, obviating the requirement for the neoplastic cells to encounter external growth stimuli (106,107). In general human cancer and thyroid cancer cells, somatic *RAS* mutations seem to be an early event. These activating mutations are frequently found in follicular thyroid carcinomas and occasionally papillary thyroid carcinoma (108).

Three members of the *RAS* family, *K-RAS* (around 85% of total), which is ubiquitously expressed, *N-RAS* (about 15%), and *H-RAS* (less than 1%), are commonly found to be activated by mutation in human tumors (109). These point mutations all prevent GAP induced GTPase activity, leaving RAS in its active, GTP-bound form. GAP deletion also leads to a similar resultant RAS activation; *NF1* or neurofibromin

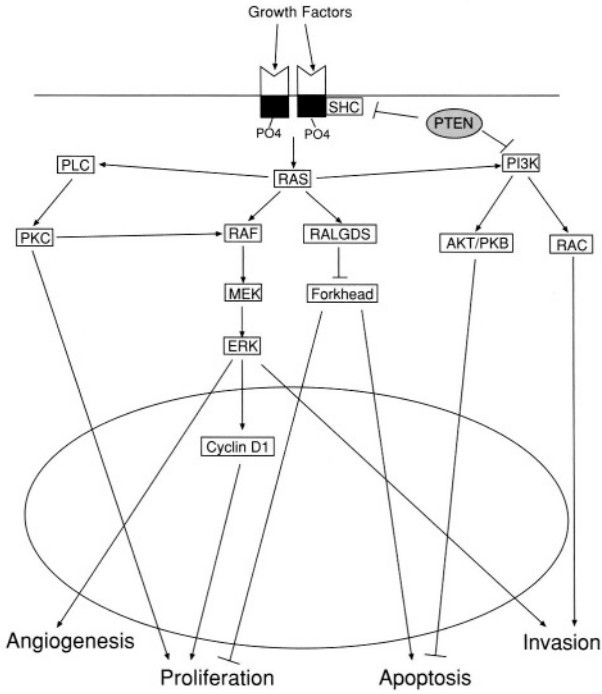

Figure 4. Downstream Mediators of *RAS.* The RAS family of proteins lead down multiple signal transduction networks to not only effect a mitogenic stimulus, but also to provide other important cellular capabilities important for cancer cells. These signaling pathways can lead to cell survival, angiogenic potential, and invasion.

loss is an example of this phenomenon and leads to benign and occasionally malignant tumors of neural crest origin (110). These single point mutations in *RAS* contribute to many of the "acquired capabilities" of cancer cells, including dysregulated growth, inappropriate survival, invasiveness, and angiogenesis (111).

In many cancers that lack *RAS* mutations, downstream effectors of RAS signaling are frequently altered, leading to acquisition of a similar set of neoplastic attributes (105). Mutations of the *BRAF* gene were initially found to be present in around 66% of melanomas and also approximately 12% of colon cancers (112). Recently, two unique somatic mutations of the *BRAF* gene have been identified in papillary thyroid carcinoma (113,114), and they offer genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway (113). Amplification of the *P110α* gene results in *PI3K* activation in 40% of ovarian tumors, while one of its downstream targets, AKT2, can also be amplified in breast and ovarian carcinomas (115). Finally, the *PTEN* tumor suppressor gene, acts as a phosphatase on specific downstream targets of PI3K, such as AKT, inactivating that pathway; *PTEN* deletions occurs in 30–40% of human cancers (116). Altogether, in human cancers the RAS proteins are not only

central mediators of both upstream growth factor receptors, but also their downstream targets play critical cellular roles, bestowing constitutively active mitogenic signals as well as multiple other important functions for oncogenesis.

Telomeres and telomerase

Telomeres are terminal structures at the ends of each eukaryotic chromosome and are composed of guanine rich, DNA 5′-TTAGGG-3′ repeats, as well as multiple DNA-binding proteins (117,118). At the end of each telomere is a single stranded 3′ overhang (119–121) that forms a large secondary loop structure, termed a T-loop (122). Telomeric DNA is maintained by telomerase, a RNA-dependent, DNA polymerase (123). Telomerase is composed of multiple subunits, two of which are crucial for enzymatic function, the RNA component (hTERC) and catalytic component (hTERT) (124) hTERT is the rate limiting component of the holoenzyme, as hTERT expression is restricted solely to cells that demonstrate telomerase enzymatic activity (125).

One of the main functions of telomeres are to protect the ends of chromosomes from forming illegitimate fusions, which would lead to genetic instability (126–128). Many DNA damage-associated proteins, such as the MRE11 complex (129) and Ku 70/80 (130,131) bind to telomere associated proteins. Thus, it has also been hypothesized that the telomere may serve as a cap, guarding the chromosome end from recognition as damaged DNA (132,133).

Both telomere length and maintenance are associated with human cell lifespan, genetic instability, senescence, immortalization, and transformation. In approximately 90% of human tumors, telomere maintenance and replicative immortality may be achieved through activation of telomerase; the remaining tumors may be maintained through "alternative lengthening of telomeres" (ALT), a telomerase-independent mechanism (134). Interestingly, studies examining malignant transformation in ALT cells lacking *P53* and *RB* function, but expressing oncogenic RAS, confirm that malignant transformation is impossible even with stable telomere lengths unless hTERT is ectopically introduced (135). Thus, 3′ overhang and T-loop maintenance by hTERT may have a role in the mechanism of transformation (136). Additionally, hTERT itself may serve some physical capping function that may be important for malignant transformation. Finally, it remains possible that hTERT has some either direct or indirect role in regulation of other important gene(s) that are critical for transformation.

In thyroid cancer, the correlation of telomere length to telomerase activity is poor, implying that there are other mechanisms that regulate telomere dynamics (137). However, most thyroid cancer cells do have sustained telomere length and have assayable telomerase activity while telomerase negative cell telomeres are likely maintained through ALT (138). Thus, similar to other types of cancers, telomere length is also important for thyroid cancer; although hTERT function and telomerase activity in thyroid cancer require further delineation of their mechanism(s) in cancer pathogenesis.

GENETIC INSTABILITY

Although the above discussed molecular alterations and their regulatory pathways are crucial to the development of a neoplastic cell, one additional hallmark may be

necessary to achieve a malignant state. This cardinal feature is genetic instability, which likely allows a cell to more rapidly acquire additional neoplastic attributes through the stepwise accumulation of mutations.

When the homeostatic mechanisms that guard the integrity of chromosomes are disrupted, additional genetic alterations may accumulate that lead to further deleterious effects. Though the various components of DNA damage detection, signaling, and repair mechanisms (139) are poorly understood, the operation of this repair machinery is incompletely understood, the adequate operation of this repair machinery is integral in preventing the survival of aberrant cells with neoplastic potential. An abnormal level of genetic instability is consistently found in many tumors (140). This instability, however, can be at either a DNA sequence level or at the level of the chromosome, in the form of aneuploidy.

Cytogenetic deformities are not an absolute finding in human cancers, as subtle DNA sequence changes can occasionally suffice to predispose to tumor formation. For instance, mutations in DNA mismatch repair genes, such as *MSH2* or *MLH1* (141), give rise to instability at the nucleotide sequence level since common replication errors can no longer be properly repaired. These tumors demonstrate microsatellite instability, which is detectable as short DNA sequence repeats seen scattered throughout the genome (142,143). These markers identify tumors that typically have two to three times as many single nucleotide mutations as compared to normal cells or cancers of the same histology but are mismatch repair proficient (144,145)

Different sets of proteins recognize and repair various types of physical DNA lesions. For instance, ultraviolet light induces adjacent pyrimidine dimerization, affecting DNA transcription and replication. Such events are repaired by the nucleotide excision repair proteins (NER) (146). In contrast, double-strand breaks (DSBs) develop in response to ionizing radiation, oxidative stress, or the stalling of replication forks at sites of DNA damage (147). These DSBs can only be repaired by homologous recombination or non-homologous end-joining (148). Thus, the cell must have unique mechanisms to recognize low levels of DNA damage at any location in the genome and shuttle the specific repair proteins required for that type of lesion. Examples include the *Xeroderma pigmentosum* group C protein involved in NER (146), MUTS proteins which bind to mismatched bases (149), and the Ku heterodimer which binds to DSBs (150). If these repair mechanisms are not in proper order, a dividing cell could improperly segregate, and depending on the type of lesion, possibly result in aneuploidy.

One important feature for a DNA damage response is the slowing or arrest of the defective cell at specific DNA damage checkpoint (151,152). This serves to delay important cell cycle transitions until repair has occurred. In human cells, the *ATR/ATM* signaling network, which can together detect a wide variety of DNA lesions through genomic surveillance during DNA replication, has a large role in this action. *ATR* disruption is lethal; *ATM* defects are not, although they are responsible for ataxia telangiectasia, which causes hypersensitivity to agents causing DSBs and increases cancer risk (152) *ATR,* when necessary, is likely to be the initiator of a global DNA damage response by activating downstream proteins like CHK1, CHK2, and RAD53. This leads to cell-cycle arrest, chromatin modulation, and further upregulation of other repair pathway proteins (139).

Alterations in DNA damage pathways, conferring genetic instability, may be early events in tumorigenesis, as is the case in microsatellite instability tumors. A heterogeneous population of cells will undergo a selective process in regards to instability. Cells with excess instability accumulate increasing amounts of DNA damage with continued proliferation eventually surpassing the threshold of viability, succumbing to apoptosis. Other cells with either too little or no genetic instability, halt at the natural barriers to immortalization. Certain cells with the appropriate amount of instability develop a survival and proliferative advantage by selecting out the right set of mutations, typically an accumulation of oncogenes and tumor suppressor genes that are now no longer able to be repaired (153). This eventually leads to clonal outgrowth and tumor formation.

ANGIOGENESIS, INVASION, AND METASTASIS

Although the initiating event in oncogenesis is not reliant upon angiogenesis, the continued success and maintainence of a tumor depends upon the utilization of this system for sustenance and eventual dissemination. In general, a solid tumor cannot grow successfully beyond 2 mm in diameter without neovascularization through the switch to the angiogenic phenotype (154,155). Thus, a tumor must acquire its own blood supply by developing new vascular structures that connect directly with existing host vasculature.

Angiogenesis is achieved by the secretion of proangiogenic factors, namely vascular endothelial growth factor (VEGF), angiopoetins, and basic fibroblast growth factors (bFGF); alternatively, the down-regulation of antiangiogenic proteins, such as endostatin, angiostatin, and thrombospondin-1 (TSP-1), also have a similar effect (155,156). These proteins signal to a complex array of downstream signaling proteins that cooperate to facilitate the overall regulation of angiogenesis. Unfortunately, this molecular circuitry remains poorly understood at this time.

An example of how tightly tumor angiogenesis may be tied to the other crucial pathways involved in tumorigenesis, is the intricate involvement of the p53 protein. Not only is p53 involved in G1 cell cycle regulation and apoptosis, but it also has a role in the regulation of angiogenesis, mainly through its interactions with TSP-1. The wild-type p53 protein can act on *TSP-1* promoter sequences and stimulate endogenous TSP-1 production (157). Since TSP-1 is a potent inhibitor of angiogenesis, wild-type *P53* gene expression loss, coincides with the switch to the angiogenic phenotype. Additionally, mutant p53 cells have been shown to upregulate VEGF (158), perhaps the most potent proangiogenic agent.

Invasion and metastasis are the final steps in tumor progression, but a unified set of responsible genetic elements has yet to be identified. Studies have described target genes that may be intimately involved in tumor migration, such as matrix metalloproteinases (159), however, the discovery of integral pathways to invasion and metastasis require further study. Just as studies have led to the discovery of common pathways for cell cycle progression, apoptosis, and autonomous growth stimulation, the discovery of a "metastasis pathway" could have utility for better understanding and treatment of cancers.

BEYOND CANCER GENOMES

To date, genetic alterations are still the easiest changes in cancer cells to detect and study experimentally. In the future, successful sequencing of an entire human cancer cell genome will certainly yield more important information for the further study of cancer. However, even with this data, many important alterations will be missed, since not all changes occur at the DNA sequence level and are instead occurring at a non-genetic level. For example, both epigenetic phenomena and post-translational modifications have critical roles in the regulation of important cellular capabilities that contribute to the neoplastic phenotype.

Epigenetic alterations

Alterations in gene expression that do not involve mutations of DNA sequences are epigenetic events. These arise during cell development and proliferation and serve as an additional method of adaptation to environmental and selective pressures. It has become clear in recent years, that epigenetic changes have an impact to the development of human cancers through silencing of tumor suppressors and DNA damage elements, chromosomal instability, and even activation of oncogenes (160,161).

Hypermethylation-mediated silencing of tumor suppressor genes may be important for tumor development since, among other advantages, it tends to lead to a selective cellular growth advantage (160,161). Methylation of cytosine residues at CpG dinucleotides occurs, and 70–80% of these dinucleotides are heavily methylated in human cells (162). Long GC-rich stretches of DNA in the human genome, termed CpG islands, are often uniquely associated with flanking genes and are protected from modification (163). These normally unmethylated CpG islands may become methylated in cancer cells, resulting in loss of expression of the flanking genes (160). This form of methylation-induced silencing affects tumor suppressors genes such as *CDH1* (164) and *P16*(165), both implicated in cancer development. Epigenetic alterations found in familial and non-hereditary forms of breast and colon cancer offer further supportive evidence for the role of methylation in neoplastic formation (166).

Although the exact mechanism of abnormal epigenetic changes leading to neoplasia is unknown, likely candidates include changes in expression of the key enzymes that regulate DNA methylation, such as the DNA methyltransferases (DNMTs). Overexpression of *DNMT* mRNA levels have been found in malignancies of various histological origin, including lung (167), colon (168,169), and ovarian (170) cancer cells. Further evidence is the fact that overexpression of DNMT1 leads to de novo methylation of CpG islands (171), and can facilitate cellular transformation (172,173).

CONCLUSIONS

The development of human cancer is a complex process that entails the alteration of multiple cell physiologic functions. Although possibilities for genetic and/or epigenetic alterations are innumerable, common principles have recently been delineated that ensure the success of any cell exhibiting a malignant phenotype. The specific pathways

and principles discussed above are known to contribute in an intimate manner to this process, but the foundation is just being set.

At this time, the study of cancer is frequently performed through experimentation with artificial cell lines or genetically altered and transformed primary cells. Although useful, it is still impossible to know the exact conditions and order of events that occur *in vivo* during metamorphosis to a neoplastic cell. Perhaps, in the future, when better cancer cell models are developed, we will define cancer by a set of distinct pathways intersecting with common principles.

Thyroid carcinomas, in particular, offer excellent models for studying cancer in general, as they offer a broad spectrum of tumor subtypes. For instance, both papillary and follicular tumors tend to be well-differentiated and may have utility in studying early genetic lesions involved in neoplastic formation. Anaplastic tumors offer the other end of the differentiation spectrum. Medullary thyroid carcinomas are associated with the MEN2 familial syndrome and supply another model to study genetic predisposition to cancer.

In the following chapters, the specific molecular defects involved in thyroid cancers will be discussed in detail. These defects may be specific for different subsets of thyroid carcinomas, but they typically lead into unifying pathways that phenotypically result in specific "acquired capabilities" for the cells. It is important to recognize these molecular changes, not only to gain greater understanding of the origin of thyroid cancers, but also to use this information for superior drug development and treatment.

Early stage thyroid cancer is standardly treated and often cured by surgical resection. However, advanced stage disease is still incurable, and current treatment measures with chemotherapy have not been shown to significantly improve morbidity or mortality. Thus, identifying these important molecular changes may one day lead to the development of new targeted therapies that can be readily tested in the metastatic setting.

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