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Oncogenic transformation and experimental models of human cancer

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1. ABSTRACT

Tumorigenesis occurs when cells undergo a series of genetic and epigenetic events that upset the balance of cell death, proliferation and differentiation. In a few cases, alterations in key regulatory steps have been identified, facilitating the design of rational cancer therapies. However, the karyotypic complexity exhibited by most solid tumors makes it challenging to identify the lesions underlying specific tumor phenotypes in most cancers. Work from many laboratories indicates that the acquisition of the tumorigenic phenotype requires several cooperating events and that a finite set of genetic alterations suffices to transform cells derived from numerous different lineages. Experimental models derived from the manipulation of oncogenes, tumor suppressor genes and telomerase provide useful platforms to delineate pathways involved in cell transformation, to connect specific cancer-associated mutations with particular cancer phenotypes and to discover and validate new targets for therapeutic development. Here we review the development of such experimental models and recent work combining such model systems with increasingly powerful genetic and chemical tools to identify and validate genes involved in malignant transformation.

2. INTRODUCTION

Tumorigenesis occurs by the accumulation of lesions affecting tumor suppressor genes and oncogenes that permit the acquisition of abnormal phenotypes such as uncontrolled proliferation, evasion of apoptosis, neoangiogenesis and metastasis. Karyotypic analyses and more recent systematic efforts to profile genetic alterations in cancers have identified a large number of chromosomal aberrations including amplifications, deletions and mutations present in human cancers. The use of high-throughput sequencing analysis, array comparative genomic hybridization (CGH) and high resolution single nucleotide polymorphism (SNP) arrays will provide the means to generate comprehensive views of these copy-number and sequence alterations (1, 2). In some cases these analyses have identified specific genetic lesions that drive tumorigenesis in specific types of cancer (3-6).

In parallel to these studies, the application of whole genome views of gene expression has proven useful in stratifying cancer subtypes and identifying signatures that predict prognosis beyond traditional pathological markers (4, 7). Together these studies are beginning to provide a comprehensive landscape of the pathways altered

in tumorigenesis. The caveat of many of these approaches is that tumors large enough to detect and biopsy are often late stage or grade, highly genetically unstable and heterogeneous. Therefore defining the events sufficient and necessary for tumorigenesis can often be complicated using these genomic approaches to define cancer genome structure.

Cell lines have frequently been used as a pliable tool for the investigation of pathways involved in tumorigenesis. Unlike tumors, cell lines are generally clonal and therefore it is much easier to examine their underlying genetic abnormalities (8). However, the majority of cancer cells lines are extracted from metastatic lesions that may have already acquired numerous mutations and may differ significantly from the initial tumor. In particular, the process of adaptation to culture likely selects for phenotypes important for *in vitro* cultivation (8-10). These factors make it difficult to determine the lesions that initiated transformation.

Another approach to identifying events involved in tumorigenesis has been to create tumorigenic cell lines from normal cell lines (11). Early approaches used sublethal doses of chemical carcinogens and irradiation to bypass cellular barriers to transformation and convert normal human cells into cancer cells (8). Alternatively, the introduction of viral oncogenes in adjunction with long term *in vitro* cultivation allowed the outgrowth of rare immortalized clones (12-16). However, these methods selected for an unknown number of mutations and therefore did not provide an ideal system in which to study events involved in transformation. Recent advances in our understanding of the pathways that regulate cell lifespan and in the technologies to manipulate genes in primary cell lines has permitted the development of experimental models of transformation with defined genetic constitution (17). These cells lines provide an experimentally tractable platform for cell-type specific dissection of the events involved in transformation.

3. GENETICALLY DEFINED MODELS OF TRANSFORMATION

3.1. A historic perspective of cellular models of transformation

Early evidence that transformation is driven by defined number of genes came from the discovery that avian and murine retroviruses contain proto-oncogenes related to normal cellular genes (18). Following this finding, many genes were discovered encoded in the DNA of viruses. These oncogenes resembled cellular counterparts including a wide range of proteins involved in proliferation and differentiation (19). Similarly, transfection of genomic DNA derived from human tumors or from chemically transformed rodent cells led to the identification of single oncogenes that were able to transform NIH 3T3 cells, a line of immortal murine fibroblasts (20, 21). These oncogenes included growth factors, growth factor receptors, components of signal transduction pathways and transcription factors. Subsequently, alterations in these genes were detected in spontaneously arising tumors where they are activated by mechanisms such as promoter hypomethylation, mutation, gene amplification and chromosomal translocation.

Although these initial oncogenes were identified by their ability to transform immortal rodent cells, further studies showed that the introduction of single oncogenes alone rarely induced the transformed phenotype in primary cells. Instead, combinations of two oncogenes, such as *myc* and H-*ras* or E1A and H-*ras*, led to morphological and functional transformation (22, 23). These observations provided the first example that oncogenes cooperated to transform cells. Eventually, several combinations of cooperating oncogenes were found that lead to rodent cell transformation (24). However, unlike rodent cells, the introduction of these same combinations of oncogenes into human cells led to cells with a limited cell lifespan that were not tumorigenic, suggesting that additional steps beyond those required to transform rodent cells were necessary to convert human cells to tumorigenesis (25). These studies suggested that immortalization is an essential aspect of cell transformation.

More than forty years ago, Hayflick determined that normal human cells have a limited proliferative capacity in culture in comparison to cancer cell lines (26). This observation suggested that alterations in the mechanisms that regulate cell lifespan are altered in human cancer cells. Following a defined number of population doublings, untransformed cells undergo a process known as senescence (27). Senescence is a cell state characterized by arrested proliferation and overt changes in cellular phenotype and varies in cells extracted from particular species and tissues. More recently, senescence has been attributed to cell physiologic insults, such as oxidative stress and DNA damage (28-30), suggesting that senescence serves as a general mechanism to suppress malignant transformation (27, 31, 32). If cells sustain mutations that permit them to bypass senescence they hit a second regulatory barrier termed crisis, which results in widespread cell death of the majority of the culture. Rodent cells can spontaneously bypass these barriers to become immortalized. Unlike their rodent counterparts, human cells rarely undergo spontaneous immortalization. Recent work has defined the molecular events that allow human cells to bypass senescence and crisis to become immortalized (Figure 1) (33).

3.2. Immortalization of human cells

3.2.1. Bypass of Senescence

The serial cultivation of primary human cells eventually leads to a phenotype known as senescence. Senescent cells are characterized by an irreversible proliferative arrest and gross changes in morphology yet remain metabolically active (34). Senescent cells display specific morphological and biochemical characteristics including increased expression of p21, $p16^{INK4A}$, senescence-associated beta-galactosidase staining and changes in chromatin architecture (35-40).

Human fibroblasts are the most robust model to examine senescence due to the relative ease that such cells

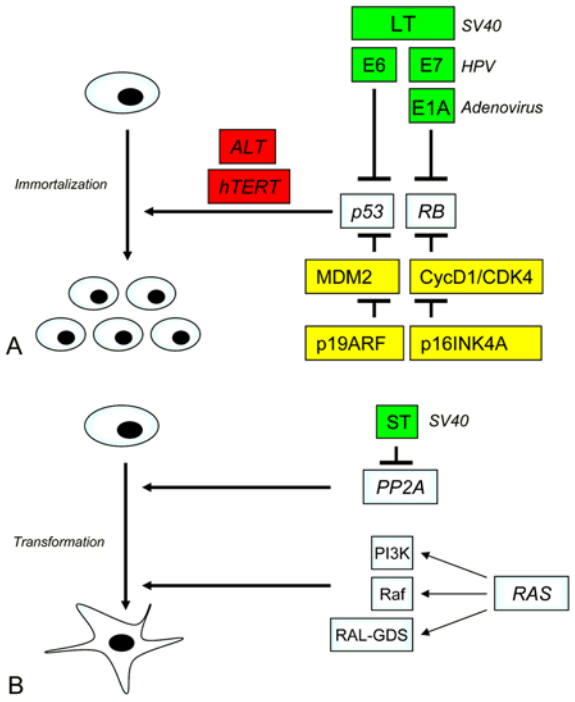

Figure 1. Pathways that cooperate to bypass senescence and crisis. A. Events required for immortalization. Maintenance of telomere length by hTERT in cooperation with disruption of the RB and p53 tumor suppressor pathways allows cells to bypass senescence resulting in immortalization. The RB and p53 pathways are inhibited by viral oncoproteins including SV40 LT, HPV E6 and E7 proteins as well as the E1A protein of human adenovirus type 5. B. Events required for transformation. Generation of transformed cells from immortalized cells requires the perturbation of PP2A by SV40 ST and RAS. RAS triggers either one or a combination of effector pathways including PI3K, RAF-MEK-ERK and RAL-GDS.

dapt to culture conditions. A variety of other types of human cells, such as endothelial, retinal pigment epithelial and mesothelial cells also exhibit a defined number of population doublings prior to their entry into senescence (26, 41, 42). However, most cell types fail to adapt to culture conditions as easily as fibroblasts. Human mammary epithelial cells, for example, undergo early proliferative arrest and require loss of $p16^{INK4A}$ to bypass this early arrest prior to senescence, and most primary epithelial cells adapt poorly to culture and arrest within a few doublings (42-46).

The introduction of the simian virus 40 large T antigen (SV40 LT) or the E6 and E7 proteins of the human papillomavirus (HPV) into many types of pre-senescent human cells permits such cells to bypass senescence. These viral oncoproteins bind to and inactivate the p53 and the retinoblastoma (RB*)* tumor suppressor proteins (47-50), suggesting a common mechanism that permits cells expressing these viral oncoproteins to bypass senescence. More recently, the suppression of *RB* and p53 by RNA interference or inactivation of these pathways through the use of dominant interfering mutants allows cells to bypass senescence (51), suggesting that these two tumor suppressor pathways contribute directly to the regulation of senescence. In addition to *RB* and p53, telomere maintenance also regulates the onset of senescence. Depending on cell type and stimulus, these three pathways function either individually or together to regulate senescence. Aberrant regulation of these pathways is detected in an array of cancers suggesting that the bypass of senescence provides an essential barrier to the development of malignancy (27, 31, 32).

3.2.2. Telomerase

Telomeres are structures that protect the ends of the chromosomes from degradation, recombination and end-joining reactions (52). Telomeres consist of large arrays of TTAGGG repetitive sequences of non-coding DNA that are progressively lost from the end of the chromosome with cell replication (53, 54). Therefore telomeres act as a determinant of cell replicative lifespan (55). The mechanism by which telomere attrition triggers senescence remains undefined although hypotheses include DNA damage signaling from short telomeres, telomere position effects and erosion of the single stranded overhang (56-60).

Telomeres are maintained by the reverse transcriptase, telomerase. Active telomerase is composed of the catalytic protein subunit (hTERT), the telomerase RNA (hTR/TERC) subunit and the dyskerin molecule (61). Telomerase uses the ubiquitously expressed TERC subunit as a template, to add TTAGGG repeats to telomere ends (62-64). Studies investigating the expression levels of hTERT revealed that the majority of human cancer cells (85%- 90%) express high levels of the enzyme in comparison to their normal counterparts (65-67). The introduction of hTERT into a variety of cells including fibroblasts, endothelial and mesothelial cells allows them to bypass senescence and facilitates the immortalization of a wide range of cell types (41, 42, 44). These observations indicate that telomere maintenance plays an important role in regulating the onset of senescence in cultured human cells.

Mammary, airway, and prostate epithelial cells as well as keratinocytes require additional alterations in addition to the ectopic expression of hTERT to bypass senescence, suggesting that other mechanisms also contribute to the regulation of senescence in these cell types in culture (42, 44, 46, 68). Since a wide variety of stimuli, including the activation of oncogenes and exposure to agents that induce cell stress, also induce a similar replicative arrest in human cells, these observations indicate that several pathways regulate entry into senescence. Consistent with this view, cells that express markers associated with senescence in cultured cells have been identified in both pre-malignant and malignant lesions (69-72). All of these stimuli, including the senescence induced by telomere attrition, are associated with sustained activation of the pathways that normally respond to DNA damage, suggesting that this replicative arrest serves as a

general mechanism to prevent the proliferation of damaged cells.

3.2.3. RB

RB was initially identified as a tumor suppressor gene, which undergoes loss of heterozygosity (LOH) or inactivation of the *RB* gene on chromosome 13q14 in spontaneous or familial forms of retinoblastoma in children (73-75). Functional inactivation of *RB* by deletion, methylation or mutation also occurs in osteosarcomas, small cell lung carcinomas and a variety of brain tumors (76, 77). In addition, inactivation of RB by the E7 protein of HPV occurs in over 90% of cervical cancers (78, 79). In normal cells, RB functions as a negative regulator of cell cycle by repressing the E2F family of transcription factors preventing the transcription of a large spectrum of proteins involved in G1-phase cell cycle progression and suppression of apoptotic cell death (80, 81). A variety of stressful stimuli including sub-optimal culture conditions, DNA damage, oncogene expression and telomere dysfunction have been suggested to induce senescence through RB-mediated pathways leading to the suggestion that RB plays a role in stress-induced senescence (45, 55, 82, 83).

Pathways involved in the direct regulation of RB are disrupted in a wide array of human cancers. Amplification of CyclinD1 and/or its partner CDK4 induces constitutive hyperphosphorylation of RB, inactivating RB and allowing cells to bypass the G1 checkpoint (84). Amplifications of these genes occur frequently in breast cancers, B cell lymphomas and squamous cell carcinomas (85). Loss of the CyclinD1/CDK4 inhibitor, $p16^{INK4A}$, has also been reported in numerous tumors including breast carcinomas, hepatocellular carcinomas and melanoma (85-87). Therefore suppression of RB activity or alterations in upstream signaling events regulating its activity appears crucial to cellular transformation and lesions in these pathways can be found in a variety of solid and hematological malignancies (Figure 1A).

The SV40 LT binds to and inactivates p53 and RB. Expression of LT permits most cell types to bypass senescence and continue to proliferate (47). Consequently, it was found that dominantly acting negative mutants of or shRNA-mediated suppression of p53 and *RB* could replace the function of SV40 LT in bypassing senescence (50, 51). Subsequent studies showed that disruption of the RB pathway sufficed to permit cells to bypass senescence. For example, expression of a CyclinD1/CDK4 fusion protein in conjunction with loss of p53 activity permitted many types of cells to bypass senescence. Additionally, loss of $p16^{INK4A}$ could replace cyclinD1/CDK4 expression and allow the bypass of senescence in conjunction with loss of p53 function (50). However, not all cell types require the inactivation of both the p53 and RB pathways to bypass senescence. For example, human mammary epithelial cells (HMECs) and keratinocytes (44, 83, 88), when propagated in the presence of either a feeder layer or after suppression of $p16^{INK4A}$ are immortalized by expression of hTERT (44, 45, 83, 88). In addition, some keratinocytes have been shown to require expression of hTERT and CDK4 to bypass senescence (89). In summary, loss of RB appears to be essential step in the immortalization of numerous cell lines, however further experiments are required to fully understand the cell-type differences in immortalization requirements.

3.2.4. p53

p53 is a tumor suppressor gene that integrates multiple signals that regulate survival and proliferation. *TP53* mutations or deletion have been detected in approximately 50% of cancers (90). Mutant alleles of p53 are directly responsible for the development of the Li-Fraumeni familial cancer susceptibility syndrome. Li-Fraumeni syndrome kindreds show a predisposition to the development of numerous malignancies including adrenocortical carcinomas, sarcomas, breast cancer and lung cancer (91). Functionally, p53 induces cell cycle arrest in response to both DNA damage and metabolic stress. In the case of excessive damage or stress, p53 regulates programmed cell death by apoptosis. Loss of this functional activity of p53 can lead to the prevention of apoptosis resulting in the accumulation of genetic damage and proliferation (92). Loss of p53 activity in primary human cells was found to delay senescence and increased levels of phosphorylated p53 and p53-related transcriptional activity is detected in senescent cells providing evidence for a direct role of p53 in this process (93-96).

Multiple signals converge on p53 to regulate its activity (Figure 1). Genes regulating these signaling pathways are frequently mutated in cancer. Upstream regulators of p53 include the ubiquitin ligase, murine double minute 2 protein (MDM2), and its antagonist, ARF (97-99). Activation of ARF is induced by high levels of mitogenic stimuli or the activation of oncogenes and leads to inhibition of MDM2 and increased expression of p53. ARF is located at the same locus as $p16^{INK4A}$ (10) and is deleted in numerous tumors including bladder carcinoma, non-small cell lung cancer, melanoma and glioblastoma (86).

3.3. Bypass of CRISIS

3.3.1. Telomerase

Experimentally, deregulation of the RB and p53 pathways permits cells to bypass senescence. As such post-senescent cells proliferate, telomere attrition continues to occur until cells enter crisis, a state characterized by widespread cell death, continued cell proliferation and karyotypic instability (100). Approximately, 1 in 10^7 cells survives crisis (47). Cells that bypass crises generally display aberrant chromosomal structures and rearrangements suggesting that genomic instability is involved in permitting cells to bypass crisis (47, 101).

Since telomere integrity is necessary to prevent chromosome ends to be recognized as fragmented DNA, the telomere shortening observed in cells that have bypassed senescence suggested that crisis is precipitated by critically short telomeres. Indeed, cells that spontaneously emerge from crisis show evidence of telomere stabilization (66, 102). Moreover, introduction of the telomerase

catalytic subunit hTERT into post-senescent human cells permits cells to avoid crisis and inhibition of telomerase activity by expression of a dominantly acting, catalytically inactive hTERT allele, dominant negative hTERT, into cancer cell lines precipitates crisis (17, 103). Taken together, these observations implicate critically short telomeres in the onset of crisis in human cells.

3.3.2. ALT (alternative lengthening of telomeres)

Although most human cancer cells exhibit stable telomere lengths with passage in culture and easily detected telomerase activity, a significant minority harbor long telomeres of heterogeneous length and lack detectable telomerase activity (104). These tumors accomplish telomere maintenance through an alternative mechanism(s) known as ALT (alternative lengthening of telomeres). Although the mechanism by which ALT maintains telomere length remains undefined, work from several laboratories suggests that ALT occurs through the exchange of sequences between telomeres (105). Indeed, recent work has confirmed that ALT cells show increased sister chromatid exchange only at telomeres (106-108).

In aggregate, these observations indicate that the regulation of cell lifespan is an important step in malignant transformation. Since alterations in the RB, p53 and telomere maintenance pathways occur in most, if not all cancers, it is likely that perturbing the mechanisms that normally regulate cell lifespan identified in cell culture contributes directly to the genesis of true human cancers. It is important to note that initial tumor formation may require only a subset of these pathways to be perturbed (109, 110). In addition, specific cell lineages may require dysregulation of particular effector molecules, perhaps explaining in part the mutation of specific molecules in certain cancers.

3.4. Tumorigenic conversion of immortalized cells

Although cells in which RB, p53 and telomere maintenance pathways have been perturbed can be passaged indefinitely in culture, such cells often maintain a normal karyotype and exhibit a normal response to growth regulatory pathways. Thus, immortalization is a prerequisite to achieve malignant transformation, but additional oncogenic events are required for tumorigenesis. Recent work has begun to define these additional cooperating events required for a fully malignant phenotype. In human cells, the additional expression of oncogenic forms of RAS and the SV40 small t antigen (SV40 ST) are required to convert immortalized cells to tumorigenicity (Figure 1).

3.4.1. Ras

Mutation of RAS family members has been found to occur at different frequencies in a variety of tumor types. The RAS family consists of three highly conserved family members *H-RAS*, *N-RAS* and *K-RAS* (reviewed in (111)). Mutations in *K-RAS* are found most predominantly in human tumors including lung, pancreas and ovarian cancer among others, while *N-RAS* mutations have been detected predominantly in melanomas and myeloid leukemias. More recently, mutations of *K-RAS* have been detected at a low frequency in patients with Noonan syndrome, who are predisposed to juvenile myelomonocytic leukemia (112). Mutations in *H-RAS* are less common and have been detected at a high frequency in bladder cancers. Germline mutations of *H-RAS* have been identified in individuals with Costello's syndrome who are predisposed to specific cancers including ganglioneuroblastoma, rhabydomyosarcoma and bladder cancer (113-115).

Mutations in genes essential for the negative regulation of the RAS pathway have been detected in numerous cancers. RAS proteins are GTPases which respond to numerous extracellular growth factors by binding guanosine triphosphate (GTP) and entering an 'activated' conformation, a process which is regulated by guanine-nucleotide exchange factors (GEFs). Inactivation of RAS is mediated by GTPase activation proteins (GAP), which hydrolyze GTP thereby inactivating RAS. Mutation of the tumor suppressor gene neurofibromatosis protein-1 (NF-1), a GAP protein, has been implicated in hyperactive RAS signaling. Patients with NF-1 mutations are predisposed to neurofibrosarcoma, astrocytomas, phaeochromocytoma and juvenile myelomonocytic leukemias (116). RAS activation can trigger multiple effector pathways primarily involved in cellular proliferation (117). These include the phosphatidylinositol 3-kinase (PI3K) pathway, the RAF-MEK-ERK pathway and the Ral-GDS pathway. Mutations in these pathways, particularly PI3K are common in many cancers. In addition, *BRAF* mutations have also been detected in a variety of cancers including malignant melanomas, thyroid cancer and colorectal cancer (118-120).

Initial studies in rodent cells demonstrated that mutated *H-RAS* cooperates with oncogenes such as *E1A* and *MYC* to achieve transformation (22, 23). Recent work suggests that specific RAS effector pathways are required for the transformation of particular types of human cells (121, 122). For example, the transformation of HMECs requires the combined activation of the RAF, Ral-GDS and PI3K pathways, while transformation of human embryonic kidney epithelial cells required the perturbation of only the PI3K and Ral-GDS pathways (121). In contrast, recent studies in human embryonic kidney epithelial cells demonstrated that several combinations of genes activating the PI3K pathway and RAF-MEK-ERK pathways could substitute for H-RAS. Specifically, either a mutated form of BRAF or a constitutively active MEK allele in conjunction with active AKT induced cell growth indistinguishable from that of cells expressing H-RAS (123). Taken together, these observations demonstrate that different cell types rely on specific RAS effector pathways to become transformed.

3.4.2. SV40 ST/PP2A

In addition to RAS, initial experiments in human cells showed that the expression of the SV40 ST antigen was also necessary to induce transformation. The major SV40 ST binding protein in human cells is the serine-threonine protein phosphatase 2A (PP2A) (124). Mutational analysis of SV40 ST demonstrated that the transforming activity of ST is dependent on perturbing the

activity of PP2A (50, 125, 126). The perturbation of PP2A function by expression of SV40 ST or inhibition of PP2A phosphatase activity with okadaic acid in immortalized cells expressing RAS induces anchorage-independent growth and allows tumor formation in immunodeficient hosts (50, 126, 127)

PP2A is a heterotrimeric protein complex composed of a catalytic C subunit, a structural A subunit and a B subunit that serves as an adaptor or regulatory subunit. Two distinct isoforms of both the A and C subunits and 17 different B subunits have been described (reviewed in(128)). We have recently shown that suppression of B56-gamma subunit expression substitutes for SV40 ST in human cell transformation (126). Since many lung cancer cell lines and some melanomas lack expression of the B56-gamma subunit, these observations suggest that loss of B56-gamma may contribute to the development of some human cancers.

Several laboratories have identified mutations in the PP2A A-alpha and A-beta subunits in a subset of melanomas, breast, lung, and colon cancers (128-130). Mutations of either the PP2A A-alpha and A-beta subunits abrogate their ability to form active complexes (126, 129). In the case of PP2A A-alpha, such mutations induce a state of haploinsufficiency. Specifically, mutation of one PP2A A-alpha allele results in lower steady state PP2A A-alpha levels, which in turn, selectively depletes specific PP2A A-alpha-B56-gamma containing complexes, due to competition among the B subunits for the limiting amounts of A-alpha present in such cancer cells harboring A-alpha mutations. Thus, mutation of one PP2A A-alpha allele acts as a tumor suppressor gene.

These studies suggest that PP2A complexes that contain the B56-gamma subunit regulate substrates involved in human cell transformation. Although the substrates regulated by B56-gamma-containing PP2A complexes remain undefined, activated alleles of PI3K substitute for SV40 ST in the transformation of human cells while inhibition of PI3K inhibits SV40 ST induced transformation. The activity of PI3K in transformation can also be replaced by expression of activated versions of the downstream effectors Akt and Rac1 (131). SV40 ST mediated transformation or suppression of the expression of PP2A B56-gamma has been shown to induce constitutive phosphorylation of AKT (126). PP2A has been implicated in the regulation of PML (132), which in turn regulates AKT, suggesting that PP2A regulates AKT indirectly. However, further work is necessary to determine the specific PP2A complexes that regulate PML activity and whether PP2A B56-gamma containing complexes also regulate AKT by other means in specific types of cells.

4. GENETICALLY DEFINED MODELS OF TUMORIGENESIS AS MODELS FOR DISCOVERY

The finding that a limited set of introduced genes suffices to induce human cell transformation provides a useful tool for the discovery of interactions between the pathways involved in tumorigenesis. The introduction of hTERT, SV40 LT, SV40 ST and activated RAS converts a wide range of normal cell lines into tumorigenic cells including fibroblasts, embryonic kidney epithelial cells, HMEC, airway epithelial cells, glial cells, endothelial cells and mesothelial cells among others (133). These observations suggest that a common set of pathways participate in the transformation of most if not all human cells. However, it is also clear that particular cancers harbor specific combinations of mutations. Consistent with this observation, specific combinations of introduced genes transform different types of human cells (46, 68, 121, 122, 134). Recent studies described below now delineate specific gene combinations that transform human cells and have resulted in experimental models that recapitulate specific stages and grades of human cancers.

Although these experimental models are useful in defining the combinations of pathways that cooperate to induce cell transformation in specific types of cells, such systems are also ideal platforms for cancer gene discovery efforts. Recent work using collections of genetic tools to identify new genes involved in a specific phenotype (forward genetic screening), the phenotypic analysis of suppression single genes (reverse genetic screening) and the screening of small molecule libraries have been applied to these models to identify tissue-specific pathways required for transformation.

4.1. Cell-type specific models of transformation

To date, many different types of primary human cells have been transformed by hTERT, SV40 LT, SV40 ST and RAS (135). The introduction of mutant alleles found in specific types of human cancers permits the development of experimental models that closely resemble specific types of cancers (46, 68, 121, 134). For example, tumors formed by transformed ovarian cancer cells recapitulated many of the features of ovarian cancers, including histological appearance, gene expression profiles and activation of specific signaling pathways (133). Additional studies have suggested the presence of cell-type specific programs and cellular microenvironment contributes significantly to transformation (68, 134). Cell types that show dependency on their microenvironment for transformation include HMECs and prostate cells. Specifically, HMECs transformed by hTERT, SV40 LT, SV40 ST and RAS were found to form tumors more efficiently in the presence of Matrigel or primary human mammary fibroblasts, suggesting that the stromal microenvironment of these cells is an essential contributor to tumor growth (134). Similarly, tumorigenic prostate epithelial cells expressing the androgen receptor exhibited a luminal phenotype resembling organ-confined human prostate cancer when placed orthotopically in the mouse prostate (68). Genetically defined models also provide useful tools to identify mutations in particular genes and define response to drug treatment. For example, introduction of cancer-associated mutants of the epidermal growth factor receptor (EGFR) into human airway epithelial cells not only induced cell transformation but also rendered such cell lines sensitive the EGFR inhibitors gefitinib and erlotinib (136).

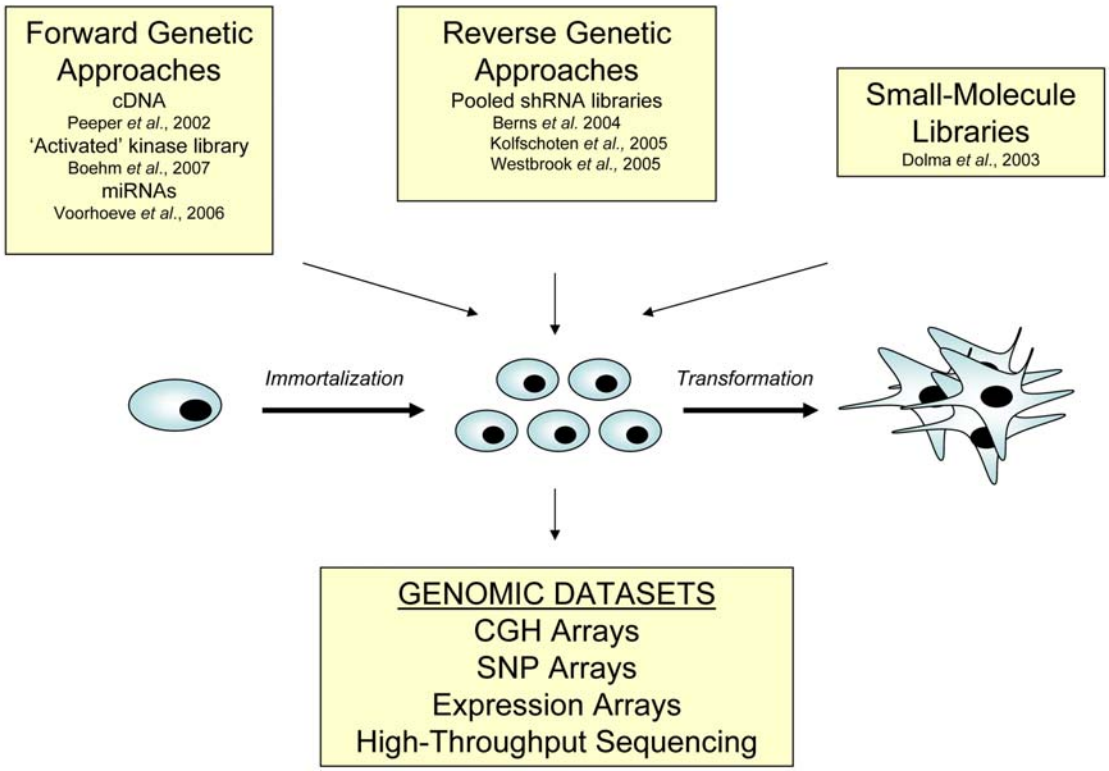

Figure 2. Discovery approaches designed to identify novel oncogenes and tumor suppressor genes using cellular models of human cancer. High throughput screening approaches include forward genetic approaches consisting of 'activated' kinase libraries, reverse genetic approaches including pooled and arrayed shRNA libraries and small-molecule libraries. Integration of putative candidates with genomic data provides validation that the candidates are relevant in human tumors.

More recently, studies from several laboratories have identified sets of genes that cooperate to transformed human cells in the absence of viral oncoproteins allowing further dissection of the pathways involved in transformation (89, 121, 122, 137-139). For example, the transformation of fibroblast cell lines required the expression of myc, H-RAS and hTERT together with loss of function of the p53, RB and PTEN tumor suppressor genes, while mammary epithelial cells were transformed by expression of RAS, myc and hTERT in conjunction with loss of p53 and RB (137). These two studies clearly demonstrated that different cell types have specific requirements for transformation. Moreover, in certain cell types myc expression was able to constitutively activate telomerase to levels that cooperate with other genetic alterations to transform human cells bypassing the requirement for hTERT expression (109, 122, 140, 141). Similarly, Chudnovsky *et al.* developed a genetically defined model of melanoma by manipulating the expression and function of hTERT, RAS and CDK4 and p53. These studies indicate that although cell transformation requires a similar general framework in most cells, particular gene combinations are required for the transformation of specific types of human cells. Taken together, these observations provide a foundation for generation of experimental models that recapitulate particular stages and grades of human tumors.

4.2. High-throughput approaches

Because the experimental models described above retain a defined genetic background, such systems provide a useful platform to identify new cancer genes. Such models can be used to study a wide range of cancer-associated phenotypes such as the bypass of senescence, bypass of crisis, anchorage-independent growth and tumor formation in immunodeficient mice. By introducing libraries of cDNAs, RNAi reagents or small molecules, one can use these systems to identify genes and pathways involved in specific phenotypes (Figure 2). In this section, we will describe recent advances in applying these approaches to cell transformation.

4.2.1 Reverse genetic screening

RNA interference (RNAi) is a conserved mechanism of gene regulation that can be exploited experimentally to regulate the expression of genes (142). Knockdown of genes can be achieved either by the introduction of short-interfering RNAs (siRNAs) by transfection or by expressing short-hairpins RNAs (shRNAs) from plasmid-based vectors. Synthetic siRNAs are readily available and have been used successfully for short-term assays. Retroviral and lentiviral expression of shRNAs provides stable knockdown of genes allowing for longer assay periods, such as those required for transformation and permit the use of RNAi in cells resistant to transfection (143-146). Although the use of RNAi is

routine for the study of specific genes, screens can either be carried out in an arrayed format in which cells are infected with a single shRNA or in pools containing a range from ten to thousands of shRNAs. Use of these complementary approaches to RNAi screening has already led to the identification of a number of tumor suppressor genes and oncogenes.

For example, several laboratories have used a pooled shRNA approaches to identified putative tumor suppressor genes that regulate p53- and RAS- dependent pathways. Berns *et al.* used a pooled screening approach to identify five genes, each of which was able to induce the bypass of p53-induced growth arrest in BJ fibroblasts immortalized with hTERT. Subsequently, these genes were validated as regulators of the p53 pathway (145). In a screen to identify genes that induce anchorage independent growth, Kolfschoten *et al.,* identified *PITX1* as a gene whose expression negatively regulates the RAS pathway in BJ cells expressing hTERT, H-RAS, SV40 ST, and lacking expression of p53 and $p16^{INK4A}$. Since low levels of *PITX1* expression were identified in prostate and bladder tumors, these findings suggest that *PITX1* is a tumor suppressor gene (147). Similarly, Westbrook *et al*. used a pooled screening strategy to identify genes whose suppression substituted for activated PI3K to induce cell transformation in HMECs expressing hTERT, SV40 LT and elevated levels of endogenous c-myc. They showed that suppression of REST substituted for activated PI3K and induced anchorage independent growth and found that 33% of colorectal cancer specimens showed evidence of deletion (148).

Lin *et al*. used an enhanced retroviral mutagen (ERM) approach to identify negative regulators of hTERT in primary human fibroblasts expressing hTERT, SV40 LT and SV40 ST. The hTERT promoter was fused to enhanced green fluorescent protein and hTERT repression was monitored by fluorescence intensity. As a proof of principle the screen identified the Mad1/c-myc pathway and SIP1, a transcriptional target of the TGF-beta pathway which have previously been described to repress hTERT (109, 149-151). In addition, they identified the tumor suppressor gene Menin as a regulator of hTERT repression. Suppressing Menin substituted for hTERT in both immortalization and transformation assays (152). Taken together, these studies suggest that these experimental models are useful as platforms for gene discovery and that that further experiments using different types of human cells transformed with different combinations of introduced genes will yield a more complete views of the genes involved in cell transformation.

4.2.2. Forward genetic screening

Complementing these loss-of-function approaches, other investigators have used forward genetic approaches to identify genes involved in cancer phenotypes. For example this approach was successfully used to identify novel genes that allow the bypass of H-RAS induced senescence in rodent fibroblasts. This screen identified *DRIL1*, a gene that was subsequently found to disrupt the RB pathway by inducing the E2F1 target Cyclin E1 resulting in the bypass of senescence (153). One of the caveats of cDNA libraries is that many genes are not activated uniquely by expression, but require additional post-translational mechanisms of modification. To address this issue, Boehm *et al*. created a retrovirally expressed cDNA library of kinases in which each kinase was fused to a myristoylation tag. Since myristoylation activates many protein kinases, the addition of this tag was predicted to induce activated versions of many of the kinases in this cDNA library. Using a cell line whose transformation depended upon constitutive AKT activity, we screened for cDNA that substituted for AKT and induced transformation. This screen identified several kinases that substituted for AKT including the non-canonical inhibitor of NF-KB signaling, *IKBKE*. *IKBKE* is amplified in 16% of human breast cancer cell lines and 30% of primary breast tumors, and suppression of *IKBKE* expression in cell lines exhibiting increased *IKBKE* copy number induced cell death. Taken together, this integrative genomic approach to defining novel oncogenes identified *IKBKE* as a breast cancer oncogene (123).

A second approach used by Voorhoeve *et al*., employed a microRNA library (miRNA) screen to identify miRNAs that cooperate with oncogenes in transformation. Individual miRNAs function to regulate the expression of large sets of genes. This screen identified miR-382 and miR-373 as oncogenic microRNA. These microRNA are predicted to decrease expression levels of the tumor suppressor gene LATS2, thereby neutralize p53-mediated CDK inhibition (154). As many miRNAs remain to be identified, further screens of this variety will be required to identify the importance of specific miRNAs in transformation.

4.2.3. Chemical screening

Genetically defined models of transformation have also been used as a system to identify drugs that target pathways involved in transformation. These models provide a platform for the identification of drugs that display synthetic lethality with transforming pathways. Dolma *et al*. used a 25,000 compound library to screen a set of cell lines transformed by different combinations of oncogenes. Expression of hTERT and either E6 and E7 or SV40 LT rendered these cells more sensitive to compounds targeting topoisomerase 2-alpha, while expression of RAS and SV40 ST increased the sensitivity of these cells to erastin a novel topoisomerase I inhibitor (155). Compounds, such as erastin, which display genotype-selective lethality, are leads for the identification of drugs with a favorable therapeutic index. Improvements the chemistry of small molecule and peptide design and the expansion chemical libraries will undoubtedly be used to find interesting targets in genetically defined models.

5. PERSPECTIVES

In summary, the creation of genetically defined models of cancer provides a platform to discover cancer genes and validate their biological functions. The introduction of cancer-associated genes into specific types of human cells has generated experimental models that

more closely resemble specific types of human cancers. The integration of these experimental models with new genetics tools and chemical genetics will facilitate the discovery of new cancer genes and the signaling networks that must be disrupted to program malignant transformation. Combined with data generated by high resolution genomic analysis and high throughput sequencing these cell lines will allow the identification of novel oncogenes and tumor-suppressor genes and biochemical dissection of the pathways they regulate.

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Abbreviations: CGH: comparative genomic hybridization, SNP: single nucleotide polymorphism, HPV: Human papillomavirus, HMEC: human mammary epithelial cells, ALT: alternative lengthening of telomeres, GTP: guanosine triphosphate, GEF: guanine nucleotide exchange factors, GAP: GTPase activation proteins, NF-1: neurofibromatosis protein-1, PI3K: phosphatidylinositol 3-kinase, PP2A: protein phosphatase 2A, EGFR: epidermal growth factor receptor, SV40 LT: Simian virus 40 Large T antigen, SV40 ST: Simian virus 40 small t antigen, CDK4: Cyclin dependent kinase 4, FISH: Fluorescence in situ hybridization.

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