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EMERGING ROLES OF CENTROSOMAL AMPLIFICATION AND GENOMIC INSTABILITY IN CANCER

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1. ABSTRACT

The carcinogenic process is multistep in terms of its etiology and multifactor in terms of its evolution. In this context, the temporal accumulation of multiple genetic changes during multistage carcinogenesis that can be mediated at least in part by genomic instability may represent crucial components of tumor cell evolution. Evidence is accumulating indicating a close link between genomic instability and cancer initiation and progression. Neoplastic cells typically possess numerous genomic lesions, which may include sequence alterations (point mutations, small deletions, and insertions) and/or gross structural abnormalities in one or more chromosomes (large-scale deletions, rearrangements, gene amplifications). Furthermore karyotypic alterations, including whole chromosome loss or gain, ploidy changes (aneuploidy and polyploidy) and a variety of chromosome aberrations are common in tumor cells. Genomic instability also involves mitotic defects associated with centrosome abnormalities. However, the question of whether abnormal centrosomes cause genomic instability or develop secondary to other changes has not been conclusively resolved. In this review, the recent studies investigating genomic instability and aneuploidy in human cancer, centrosome amplification and the role of centrosomal duplication in chromosomal mis-segregetion, and genes implicated in regulating chromosome segregation, centrosomal amplification and progression in cancer cells are discussed.

2. INTRODUCTION

It is now accepted as almost axiomatic that multiple genetic alterations must occur for a normal cell to transform into a cancer cell. A relationship between genomic damage and cancer development has been suggested since the beginning of the 20th century (1). The genetic alterations occurring in tumors can be divided into four major categories: [1] subtle sequence change; [2] gene amplifications or deletions; [3] chromosome translocations; and [4] alterations in chromosome number (2). While subtle sequence instabilities as manifested by nucleotide-excision repair-associated instability (NIN) and microsatelite instability (MIN) are rare, chromosomal instability (CIN), involving gains and losses of whole chromosomes, appear to be frequent events in most human tumors (2, 3). Intra-chromosomal genomic instability in cancer reflects an increased rate of appearance of DNA alterations in tumor cells, which may arise either from increased rates of damage overwhelming the ability of normal repair systems to restore genomic integrity, or defective repair systems being unable to cope with normal rates of damage being generated through normal cellular and environmental mechanisms. Moreover, this instability underlies the vast majority of the genomic events. In contrast, chromosomal instability at the whole chromosome level arises from inappropriate segregation, recombination and similar events, and generates relatively few genomic changes. Processes that facilitate these changes in the genome include increased rates of damage that may arise from either external or internal sources. Exogenous factors implicated in promoting genomic changes include radiation damage and damage arising from chemical agents. While highly relevant to therapy-induced secondary cancers, exogenous damage of this type cannot directly underlie the ongoing heritable genomic instability seen in cultured tumor cells. Endogenous factors actively and directly increasing genomic damage can include over expression or improper nuclease sequestration, which has been

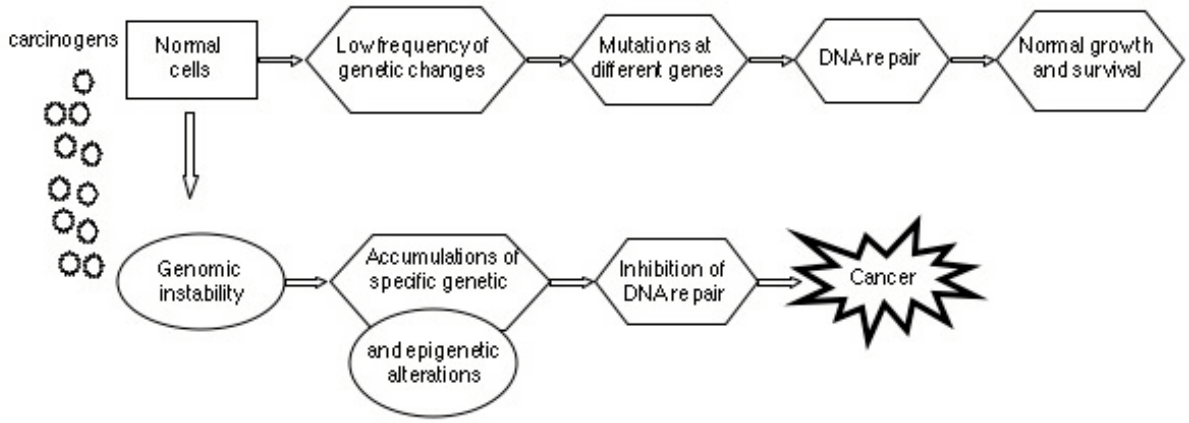

Figure1. Schematic view of how normal cells transform into a cancer cells and the impact of genomic instability on this transformation process.

demonstated in model systems, or telomere deficiencies generating bridge–breakage–fusion events (4,5). Telomere shortening is a natural consequence of somatic cell proliferation, and will continue up to that point where apoptosis is activated, unless a mutational event activating telomerase occurs.

Inefficient or defective repair that is unable to cope with normally occurring damage is well documented and provides an indirect means of generating genomic instability (6). This can arise from inherent deficiencies in the repair enzymes themselves, or from checkpoint defects, which fail to halt the cell cycle until repair can be completed. The reader is referred to an excellent recent article by Wood *et al*. for a comprehensive review of the genes involved in DNA repair (7). DNA repair is essential to preserve the fidelity of genomic information, removing damage generated by naturally occurring environmental insults as well as from the inevitable errors arising during genomic evolution of the cancer cell. The genome is particularly vulnerable during its replication, and segregating chromosomes to daughter cells provides a further opportunity for large losses and perturbations of genetic information. Damage repair handles single mutational events through base excision repair, nucleotide excision repair, or mismatch repair. Specialized genes exist for handling various forms of repair and are optimized for particular types of damage, such as large or small chemical adducts, replication fork errors, or UV-generated cytobutane pyrimidine dimers. Two genetic cancer-prone syndromes, xeroderma pigementosum and hereditary nonpolyposis colorectal cancer provide clear examples of how defects in particular repair genes can contribute to genomic instability and cancer malignancy (6,8).

3. GENOMIC INSTABILITY AND CANCER

Although suggested to be a critical factor for cancer development for more than twenty-five years (9), recent studies have revitalized the concept of genomic instability as a primary force in tumor evolution. Direct support for this hypothesis has come from studie confirming that specific mutations in DNA repair and checkpoint genes occur in multiple human tumors (10, 11). Genomic instability implies both the loss of control of regulation of chromosome structure and number, as well as the loss of other functions that allow propagation of these defects to new daughter cells (2). The spectrum of genetic alterations that occur in genetically unstable cells varies considerably. On one end of the spectrum are subtle changes in DNA sequences, such as microsatellite instability that is caused by defects in the DNA mis-match repair system. The other end of the spectrum is represented by gross chromosomal instability, characterized by defects including chromosomal breaks, amplifications or deletions of chromosomal regions, as well as gains or losses of whole chromosomes. Individual tumor cells often display complex cytogenetic abnormalities that include both numerical and structural chromosomal alterations. Given the large number of chromosomal aberrations, it is conceivable that many tumors develop chromosomal instability at an early stage, which enhances the potential for the acquisition of additional alterations in the genome during cancer progression (Figure 1). It should be noted, however, that some types of cancers such as certain malignant lymphomas and leukemias, do not display a high level of genomic instability but instead display a limited number of karyotypic abnormalities that are retained during tumor evolution. The mechanisms by which such tumors respond to selective pressures during clonal expansion remain to be elucidated.

4. ANEUPLOIDY : A POTENTIAL INDICATOR OF GENOMIC INSTABILITY

In normal cell division, each set of replicated chromosomes is usually equally distributed to each of the new progenitor cells. However, errors that lead to imbalances in chromosome segregation do occur, often with catastrophic consequences. Chromosomal instability is characterized by losses or gains of whole chromosomes (aneuploidy), as well as chromosome rearrangements. It is estimated that numeric chromosomal imbalances, are the most prevalent genetic changes recorded among

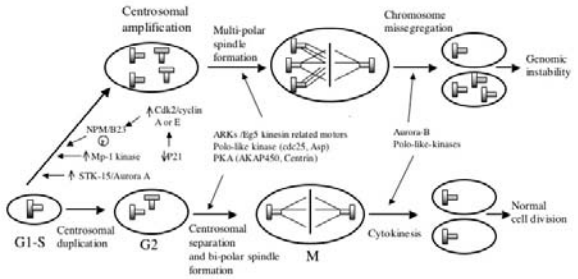

Figure 2. A working model regarding functions of G_1-S and G_2-M checkpoint proteins in the control of centrosome duplication. Under normal conditions, this results in equal segregation of chrosmosomes and normal cell divisions. Centrosomal amplification may result through inactivation of these checkpoint proteins and/ or defects in cytokinesis and eventually leads to chromosomal missegregation, aneuploidy and genomic instability.

over 20,000 solid tumors analyzed thus far (3, 12). Whether aneuploidy is a cause or consequence of cancer has long been debated (13). Several lines of evidence now argue in favor of aneuploidy as a discrete chromosome mutation event that contributes to malignant transformation and tumor progression. The universal nature of aneuploidy in most malignant tumors and in many early stages of carcinomas suggests that this condition is intimately involved in the tumorigenic process. Recent data suggest that aneuploidy develops from defects in the process of chromosomal segregation during mitosis. Aneuploidy can arise through two principal mechanisms. Cells either can proceed through a tetraploid intermediate to a multipolar mitosis that creates random chromosome distribution or they can proceed directly to aneuploidy through failure of a critical control of euploidy. In many human carcinomas, cells with tetraploid DNA content arise as an early step in tumorigenesis, preceding the formation of aneuploid cells (9, 14). Tetraploidy can arise either through disruption of chromosome segregation during mitosis (15-18) or through failure of cytokinesis (19). Alternatively, aneuploidy can arise from mechanisms independent of a tetraploid intermediate such as, loss of control of centrosome duplication, leading to abnormal centrosome amplification, multiplolar spindles and aneuploidy.

5. CENTROSOMES: THE MAJOR MACHINERY OF CHROMOSOME SEGREGATION

Centrosomes consist of a pair of centrioles surrounded by a protein matrix known as the pericentriolar material or centrosome matrix. Centrosome duplication is a critical cell cycle event. Centrosomes are duplicated once and only once during each cycle in normal cells. Duplication of the single centrosome furnishes the cell with the two organelles required to organize a bipolar spindle that segregates replicated chromosomes into progeny cells. If proliferating cells fail to coordinate centrosome duplication with DNA replication, this will inevitably lead to a change in ploidy, and the formation of monopolar or multipolar spindles which will generally provoke abnormal segregation of chromosomes. Indeed, it is well established that errors in the centrosome duplication cycle may be an important cause of aneuploidy and thus contribute to cancer development (1). This view has recently received support with the description of extra copies of centrosomes supernumerary centrosomes) in many solid human tumors including brain (20, 21), breast (21-24), lung (21), colon (21), prostate (21), pancreas (25), bile duct (26), and head and neck (27). Hematological malignancies like non-Hodgkin's lymphoma and acute leukemia also display centrosome aberrations at a high frequency (28, 29). Increased centrosome numbers have also been reported for cervical cancers that are associated with high-risk human papillomavirus (HPV-16/HPV-18) infection (30-32). Numerical centrosome aberrations are frequently accompanied by structural irregularities. These include, increase in centrosome size, the formation of acentriolar bodies and alterations in the phosphorylation status of the pericentriolar matrix compounds (33-37). Centrosome abnormalities in tumors and tumor-derived cell lines could induce two distinct phenomena contributing to tumorigenesis (21). First, all centrosomes, regardless of size, shape and number, were able to participate in the formation of structurally and functionally aberrant mitotic spindles. Second, cells with abnormal centrosomes missegregated chromosomes at a high rate producing aneuploid cells with dramatically different chromosome numbers (chromosome instability) (11).

6. CONTROL OF CENTROSOME DUPLICATION DURING CELL CYCLE PROGRESSION

In addition to their role as the microtubule organizing center in the interphase cell and in mitotic cells, centrosomes also appear to play a role in regulation of cell cycle progression itself, since microsurgical removal or laser ablation of centrioles results in failure of cytokinesis and G_1 arrest. Centrosome duplication is strictly co-ordinated with DNA replication, mitosis and cell division. Importantly, several centrosome-associated kinases and target substrates implicated in the regulation of centrosome duplication cycle become altered during the development of centrosome amplification in cancer (summarized in table 1). The detailed mechanisms by which centrosome aberrations develop in malignant tumors are largely unknown. Centrioles function as microtubule organizing centers of interphase cells and they must duplicate and separate in the G_1 phase to become the poles of mitotic spindles. Several lines of evidence suggested a role of the G_1 phase cell cycle checkpoint to ensure proper centriole duplication and separation (38-41). Accordingly, defects of G_1-S phase regulatory proteins should not only lead to unregulated proliferation, but also cause karyotypic instability by uncontrolled centrosome replication (Figure 2). The most fundamental event in the G_1-S checkpoint is the stabilization and activation of p53, which, in turn, induces transcription of the p21 (waf1, cip1, sdi1, *mda*-6) gene (42), an inhibitor of the cyclin E-cdk2 complex. The p53 tumor suppressor protein plays a central role in the decision of a cell to undergo apoptosis after exposure to diverse stresses, including DNA damage. The first hint that tumor suppressors might be relevant to centrosome biology came from the analysis of p53 knockout (p53-/-) mouse

Table 1. Centrosome-residing proteins that can control cell duplication

Function	Protein	References
Tumor suppressor proteins	p53	43
	pRB	52, 67
	BRCA1	59, 61
	BRCA2	65
Centrosome duplication	Cdk2/cyclin E or A	38, 39, 40
	p21 (waf1, cip1, sdi1, *mda*-6)	42, 45, 79
	Nucleophosmin	82
	BTAK/STK-15	106
Centrosome separation, bipolar		
spindle formation	Polo-like kinases (cdc25)	103
	Aurora-related kinases (Eg5	
	kinesin- related motor)	104, 108
	Nek 2(C-Nap1)	105
	PKA (AKAP450, Centrin)	22
Centrosome-associated		
Phosphatases	protein phosphatase 4	124
	protein phosphatase 1α	125
Cell signaling	casein kinase 1α, II	126, 127, 128
	src family kinase *fyn*	129
	PKC-theta	130

embryo fibroblasts (MEFs), which undergo centrosome amplification (43). A significant proportion (10-30%) of p53-/- MEFs cultured *in vitro* have supernumerary centrosomes. Further analysis demonstrated that these cells undergo multiple rounds of centrosome duplication during S phase. Re-introduction of wild type p53 prevents the re-duplication of centrosome (44), demonstrating that wild type p53 is required to limit centrosome duplication to a single round each cell cycle. Furthermore, supernumerary centrosome has been described following deletion of two p53 targets – the CDK2 inhibitor p21 (waf1, cip1, sdi1, *mda*-6) (45) and GADD45, and following over expression of MDM2, a p53 inhibitor. Thus, it is well established that the loss of a functional p53 pathway favors the appearance of cells with supernumerary centrosomes, both in tissue culture and in tumors (43, 46).Mutational inactivation of p53 has also been described to initiate multiple rounds of centrosome replication within a single cell cycle (43, 46-48). This effect seems to be mediated at least in part through the p53/p21 (waf1, cip1, sdi1, *mda*-6) axis, since induced expression of the cdk2 inhibitor p21 (waf1, cip1, sdi1, *mda*-6) in p53-/- cells partially restores centrosome duplication control, suggesting that p21 (waf1, cip1, sdi1, *mda*-6) is one of the multiple effector pathways of the p53-mediated regulation of the centrosome duplication cycle. Aligned with these reports, reduced p21 (waf1, cip1, sdi1, *mda*-6) expression as well as over expression of the p53 inhibitor *mdm2* results in centrosome over-duplication, gross nuclear abnormalities and polyploidy in human cells. It has been argued that loss of p53 causes centrosome overduplication within a single S phase (43, 44). However, a recent study favors an alternative interpretation (49). Over expression of Aurora-A and other mitotic kinases was shown to cause centrosome amplification by interfering with the successful completion of cell division, giving rise to cells that were characterized by both centrosome amplification and polyploidy. Remarkably, both of these phenotypes were exacerbated in a p53-/- background (49). Therefore, centrosome amplification in p53-/- cells does not necessarily imply a role for p53 in the regulation of centrosome duplication, but instead might reflect the involvement of a p53-dependent checkpoint in the elimination of cells that emerge from aborted divisions (17, 19, 49-52).

Several additional findings support the view that the absence of p53 favors the emergence of supernumerary centrosomes through an indirect, checkpoint-related mechanism. Centrosome amplification is not an inevitable consequence of p53 deficiency *in vivo* (53), indicating that the elimination of p53 is not in itself sufficient to deregulate the centrosome cycle. Furthermore, the targeted inactivation of p53 in diploid human cells did not cause aneuploidy, although it favored the formation of tetraploid cells (54). It is also interesting to consider the generation of supernumerary centrosomes by the HPV-encoded oncoproteins, E6 and E7. Whereas E7 primarily targets the retinoblastoma (RB) gene product, E6 causes the ubiquitin-dependent degradation of p53. Yet, over expression of E6 in primary human keratinocytes failed to exert a rapid effect on centrosome duplication, but instead produced centrosome amplification in conjunction with multinucleation (55, 56). Similarly, when expressed in a lung cancer cell line, E6 did not cause chromosomal instability unless mitotic-spindle formation was transiently abrogated (57). Considering that both DNA replication and centrosome duplication are regulated through the RB pathway, it is speculated that mutational inactivation of this pathway—a common event in human tumors—could provide the appropriate environment for centrosome over duplication (41). However, although the loss of RB function might create permissive conditions for centrosome over duplication, this genomic change alone is clearly not sufficient. Several rounds of centrosome duplication could only occur in RB-deficient cells if S phase was sufficiently prolonged, for instance, in response to activation of a

DNA-damage checkpoint. Studies on the E7 oncoprotein of HPV seem consistent with a role of the RB pathway in restraining centrosome duplication (56). So, the available evidence indicates that the E6 and E7 oncoproteins use distinct mechanisms for generating centrosome amplification and chromosomal instability (31). By interfering with p53, E6 might favor the survival of cells exiting aberrant mitoses. E7 might inactivate RB and thereby predispose cells for centrosome over duplication. If these interpretations are correct, then the two cooperating oncoproteins of high-risk HPV would trigger two major mechanisms for centrosome amplification.

Recent studies indicate that the BRCA1 tumor suppressor localizes to centrosomes (58, 59). Although BRCA1 has not been implicated directly in centrosome duplication, it contains a small γtubulin-binding domain (60). Over expression of the BRCA1 γ–tubulin binding domain competes with BRCA1 for γ-tubulin binding, causing a decrease in the recruitment of BRCA1 and γ-tubulin to mitotic centrosomes, and results in the formation of multipolar mitotic spindles (60). BRCA1 is normally phosphorylated during S phase, and in response to DNA damage, although the relevance of BRCA1 phosphorylation to its function as a tumor suppressor is unclear (61). Interestingly, only hypophosphorylated BRCA1 interacts with γ–tubulin, and accumulation of hyperphosphorylated BRCA1 in response to okadaic acid prevents co-immunoprecipitation of BRCA1 and γ-tubulin, reduces BRCA1 and γ-tubulin at centrosomes, and causes the formation of multipolar mitotic spindles (60). This data suggest that centrosome maturation, required for proper spindle function during mitosis, may be BRCA1-dependent (62). *brca*-/- tumors display genetic instability (63) and cells from *brca*1 mutant mice display centrosome amplification (64) and genetic instability, as well as a defective G_2/M DNA-damage checkpoint. While it remains to be documented, centrosome abnormalities caused by perturbation of BRCA1 may contribute to genetic instability. It is interesting to note that perturbation of BRCA2, a structurally unrelated tumor suppressor that forms a complex with BRCA1, also causes centrosome amplification (65) although BRCA2 has not been localized to centrosomes. It will be interesting to determine in the future if BRCA2 might have similar affects on centrosome function, or if the centrosome function of BRCA1 is independent of BRCA2.

The p53, Cdk2, cyclin A and cyclin E genes are transcriptionally controlled in late G_1, and contribute to transcriptional regulation of cell cycle genes at the G_1/S boundary when centrosome duplication occurs (66, 67). For example, the retinoblastoma tumor suppressor protein (pRB) is required for centrosome duplication through its ability to bind and inhibit the E2F family of transcription factors (66, 67). The targets of E2F include many genes whose products are required for centrosome duplication (e.g., Cdk2, cyclin A and cyclin E) (66, 67). At different points in the cell cycle, either cyclin A (41, 68) or cyclin E (69) alone are sufficient to support centrosome duplication, and misregulation of either can cause genetic instability or centrosome amplification, or both (68, 70-72). In addition, the p53 activator p14/19ARF (67) is an E2F target, and BRCA1 interacts with both the pRB and p53 pathways (61). Therefore, it is not surprising that deregulation of G_1 cell cycle control or of transcriptional control at G_1/S might also lead to defects in centrosome duplication. As examples, over expression of the HER2/neu oncogene results in constitutive mitogenic signaling and causes centrosome amplification (73), as does over expression of the p53 inhibitor MDM2 (46). In addition, deletion of the SKP2 component of the SCF ubiquitin ligase causes centrosome amplification in mice, presumably by preventing the periodic degradation of cyclin E (74).

In line with these observations, both inactivations of pRb and over expression of cyclin E have been shown to induce abnormal centrosome synthesis and genomic instability (56, 46, 71). Over expression of cyclin A, E2F-2, and E2F-3 also leads to multiple rounds of centrosome duplication (41). On the other hand, over expression of p16INK4A, p21 (waf1, cip1, sdi1, *mda*-6) and p27KIP1 is able to block centrosome duplication (40, 41, 75). CyclinE-CDK2 is required for the initiation of DNA synthesis, an event that occurs at approximately the same time in the cell cycle as centrosome duplication (76). In mammalian somatic cells, both cyclinE levels and cyclinE/CDK2 activity peak at the G_1/S transition, which is similar to the timing of centrosome duplication (77). Inhibition of cyclinE/CDK2 by the CDK inhibitor p21 (waf1, cip1, sdi1, *mda*-6) blocks centrosome duplication and depletion of CDK2 or cyclinE inhibits centriole separation activity, a prerequisite for centrosome duplication (39). Overexpression of cyclinE is frequently observed in many cancers (78) and p21 (waf1, cip1, sdi1, *mda*-6) functions as a tumor suppressor (79). One of the substrates for cyclinE/CDK2 is Nucleophosmin/B23 (NPM), which is involved in nuclear/cytoplasmic trafficking and possesses molecular chaperone properties (80, 81). NPM is associated with unduplicated, but not with duplicated centrosomes, and dissociates from centrosomes upon phosphorylation by cyclinE/CDK2 (82). Microinjection of anti-NPM antibody, which blocks this phosphorylation, suppresses the initiation of centrosome duplication. Expression of the NPM deletion mutant, which is unable to be phosphorylated by cyclinE/CDK2, blocks the initiation of centrosome duplication. When NPM undergoes hyperphosphorylation there is premature separation of centrioles and consequently centrosomal amplification results. A functional proteomic approach identified a posttranslationally modified form of NPM, most likely phosphorylated NPM (PNPM), to be upregulated during melanoma progression and elevated NPM levels have been associated with breast cancer, colorectal cancer and leukemia (83-86).

7. CENTROSOME-ASSOCIATED PROTEIN PHOSPHORYLATION AND CANCER

Cell cycle progression is interrupted when checkpoint surveillance mechanisms detect DNA damage or abnormal mitotic spindle function (87, 88, 89). During cell division mitotic checkpoints operate that monitor (90) centrosome duplication and bipolar attachment of

chromosomes to the mitotic spindle (91). These checkpoints are controlled by the activity of kinases, phosphatases, and their substrates. Centrosomes undergo an increase in protein phosphorylation at the time of G_2/M transition suggesting that centrosome separation and mitotic function are regulated by phosphorylation (92-94). Kinases known to regulate cell cycle progression have been localized to centrosomes and mitotic spindle poles. Kinases implicated in centrosome function include: p34cdc2/cyclin B, polo-like kinase 1 (PLK1), cAMP-dependent kinase (PKA), STK15 (BTAK) and Nek2 (95-106). Disruption of kinase activity leads to centrosome and mitotic spindle defects. Therefore, it is clear that centrosome protein phosphorylation is important for cell cycle progression and centrosome function.

Tumor centrosomes show activation of associated kinases and altered levels of protein phosphorylation (22, 106-108). Until recently, gross chromosomal abnormalities were thought to develop downstream of accumulated oncogenic events and not by disruption of a single centrosome- or spindle-associated proteins. However, recent studies have demonstrated that aneuploidy and genomic instability can result from over expression of a single centrosome-associated kinase gene. Experimental amplification of kinase STK15 (BTAK) and mutations in aurora both affect spindle pole behavior and result in aneuploidy or monopolar spindles, respectively (106, 109). Ectopic expression of STK15 (BTAK) is sufficient to transform NIH 3T3 cells and leads to the appearance of abnormal centrosome numbers (106). When STK15 (BTAK) is over-expressed in near diploid human breast epithelial cells, supernumerary centrosomes and aneuploidy result (106). The finding that STK15 (BTAK) is amplified in 12% of primary breast tumors corroborates these observations.

Apparent centrosome amplification has been attributed to deregulation of the various genes described above. This raises the question of whether the absence or malfunction of these different gene products produces centrosome anomalies by distinct mechanisms, or, alternatively, whether distinct primary defects converge onto a common secondary defect, which then gives rise to an increase in centrosome numbers. A common mechanism, centered on mitotic failure leading to tetraploidization, would readily explain why multiple genes, although acting in pathways presumably unrelated to centrosome duplication, all lead to a similar centrosome amplification phenotype.

8. CORRELATION BETWEEN CENTROSOME AMPLIFICATION, ANEUPLOIDY AND CHROMOSOMAL INSTABILITY

Centrosome amplification and chromosomal instability occur exclusively in aneuploid tumors and tumor-derived cell lines in contrast to diploid tumors, which contain centrosomes that are functionally and structurally normal (22, 35, 36). The degree of genomic instability parallels the degree of centrosome abnormalities in cell lines from breast (110), pancreas (34), prostate (33), colon (35), and cervix tumors (32), from short-term culture of mouse mammary tumors (73), and from SV40 ST (small T antigen) over-expressing fibroblasts (111). Centrosome abnormalities were higher in high-grade prostate tumors (33) and high-grade cervical tumors (32) than in low-grade tumors. In prostate cancer, centrosome amplification has been implicated in the development of abnormal mitoses and chromosomal instability (CIN) facilitating progression to advanced stages of the disease (33, 112). Strong support for a direct mechanistic link between centrosome amplification and CIN is suggested by the significant linear correlation between centrosome amplification and the rate of change in karyotype seen in human breast tumors (36). Although this correlation alone does not necessarily confirm cause and effect, these observations have led many authors to propose the hypothesis that centrosome amplification is the foremost cause of genomic instability observed in most tumors (111, 36, 34, 32). An alternative hypothesis has been proposed that chromosomal instability seen in cancer cells is caused by aneuploidy, that is aneuploidy itself destabilizes the karyotype and thus initiates CIN leading to widespread heterogeneity in tumor cell phenotypes (113-115).

Several independent lines of evidence support the proposition that centrosome abnormalities drive genomic instability. In a recent study of human breast tumors, all specimens of ductal carcinoma *in situ* examined showed significant centrosome amplification, while aneuploidy was present, on average, in only 35% of *in situ* breast tumors, suggesting that centrosome amplification is an early event that occurs prior to invasion in breast tumors (36). Furthermore, cells transfected to express the HPV E7 oncoprotein undergo centrosome amplification prior to developing nuclear morphology associated with aneuploidy (30, 55). Finally, in a xenograft model of pancreatic cancer, metastatic foci showed a higher incidence of centrosome amplification than did the primary xenograft, and abnormal centrosome numbers were accompanied by a higher frequency of abnormal mitoses (116). Taken together, these studies suggest that not only does centrosome amplification drive CIN, but also that this instability causes the tumor to progress to a more advanced stage.

9. CENTROSOME AMPLIFICATION AS POTENTIAL INDICATOR OF TUMOR AGGRESSIVENESS

Can centrosome amplification be utilized as an indicator of tumor progression and the potential of a cancer cell to develop aggressive tumor phenotypes? Centrosome amplification is not only characteristic of tumors in general, but also is more pronounced in advanced stage malignancies, in recurrent tumors, and in cell lines that show more aggressive malignant phenotypes in xenograft animal models (26, 27, 117). These observations suggest that centrosome amplification might be useful in monitoring tumor progression and phenotypic diversity in cancer. Finally, in association with other established prognostic factors, centrosome amplification may be helpful in predicting outcomes and survival of patients with cancer.

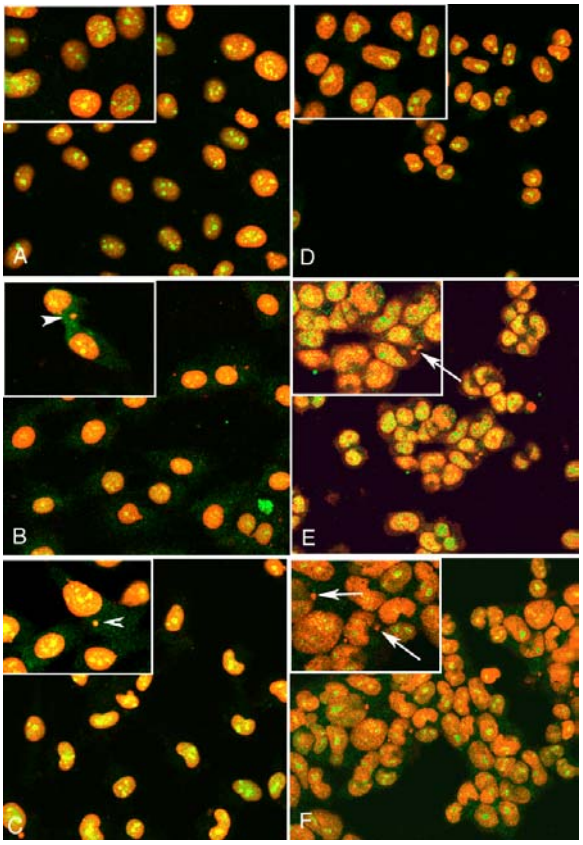

Figure 3. *PEG-3* expression induces micronuclei formation. The different cell types were stained with CREST antibody directed against kinetochore protein and an Alexa-488-labeled secondary antibody (in green) and nuclei were counterstained with propidium iodide. Images were obtained with a LSM 410 confocal microscope using a 60X objective. Arrowheads indicate CREST-positive micronuclei and arrows indicate CREST-negative micronuclei. The majority of the cells shown in this figure are in interphase. Inserts show a higher magnification view. Panel: **A**: CREF; **B**: CREF-*ras*; **C**: CREF-*PEG-3*; **D**: E11; **E**: E11-NMT; **F**: E11-*PEG-3*. (From Emdad *et al.,* 121).

10. *PEG-3* AND GENOMIC INSTABILITY

Progression elevated gene-3 (*PEG-3*), was cloned by our group in 1997 (118) using subtraction hybridization as an up regulated transcript associated with the process of transformation and tumor progression of rat embryo fibroblasts. Ectopic expression of *PEG-3* in rodent and human tumor cells markedly augments *in vitro* anchorage-independent growth and tumor cells display an aggressive cancer phenotype with increased new blood vessel formation, i.e., angiogenesis, which correlates with elevated expression of vascular endothelial growth factor (119). Moreover, *PEG-3* expression in rodent tumor cells correlates directly with genomic instability (120), as indicated by chromosomal alterations and gene amplification. Cytogenetic analysis of various rodent transformed cells demonstrates a direct relationship between elevated *PEG-3* expression and induction of chromosomal abnormalities, including an increase in the percentage of polyploid and aneuploid cells and defined chromosomal anomalies. Additionally, elevated endogenous or ectopic expression of *PEG-3* in rodent and human tumor cells, respectively, enhances gene amplification, as monitored by resistance to methothrexate and amplification of the dihydrofolate reductase gene (120).

Very recently our group further elucidated the functional significance and role of *PEG-3* in cancer progression with a specific focus on genomic instability (121). In this investigation, we endeavored to understand the mechanism underlying *PEG-3*-induced aneuploidy by assaying micronucleus formation and staining of kinetochores with CREST antibody, staining of centrosomes to define centrosomal abnormalities and analyzing the expression patterns of proteins implicated in centrosomal duplication. Immunocytochemical observations of a specific clone of Fischer rat embryo fibroblast (CREF) cells stably expressing *PEG*-3 or the Ha-*ras* oncogene document that over expression of *PEG*-3 induces loss of chromosomes, as revealed by the appearance of micronuclei in the cytoplasm (Figure 3).

Staining with CREST antibody indicated a preponderance of CREST negative micronuclei in the *PEG*-3 expressing cells. Staining of centrosomes with γ-tubulin antibody confirmed centrosome amplification in CREF cells stably transfected with a *PEG*-3 gene or expressing *PEG*-3 as a consequence of transformation by Ha-*ras* (Figure 4). Similar results were obtained with E11-NMT, a clone of mutant type 5 adenovirus (H5ts125) transformed Sprague-Dawley rat embryo cells displaying a progressed and aggressive cancer phenotype, and in E11-*PEG*-3, a clone of E11 cells transfected with a *PEG*-3 gene and displaying a progressed and aggressive cancer phenotype. Using Hela-Tet-*PEG* cell clones (which expressed *PEG*-3 as a function of treatment with doxycycline) and control tetracycline vector Hela-Tet-*Vec* clones, experiments were performed to determine if induction of rat *PEG*-3 affects genomic stability as monitored by frequency of micronuclei formation and centrosomal amplification. The percentage of micronuclei was enhanced in Hela-Tet-*PEG*-3 clones grown for several weeks in doxycycline, but not in 96-hr treated cultures (unpublished observation). However Hela-Tet-*PEG*-3 displayed a modest increase in centrosomal amplification in comparison with Hela-Tet-vec, even when grown in the presence of doxycycline for a short period of time (unpublished observation).

Since centrosomal abnormalities have been reported in a wide range of malignant tumors (discussed above), and the majority of these tumors are aneuploid, we also analyzed the expression patterns of several proteins involved in centrosomal regulation in the context of *PEG-3* over expression in rodent cells. As shown in Figure 5 western blot analysis revealed strong expression of p21 (waf1, cip1, sdi1, *mda*-6) in E11 and CREF cells. There was a mild and marked reduction of p21 (waf1, cip1, sdi1, *mda*-6) expression in CREF-*PEG-3* and CREF-*ras* cells,

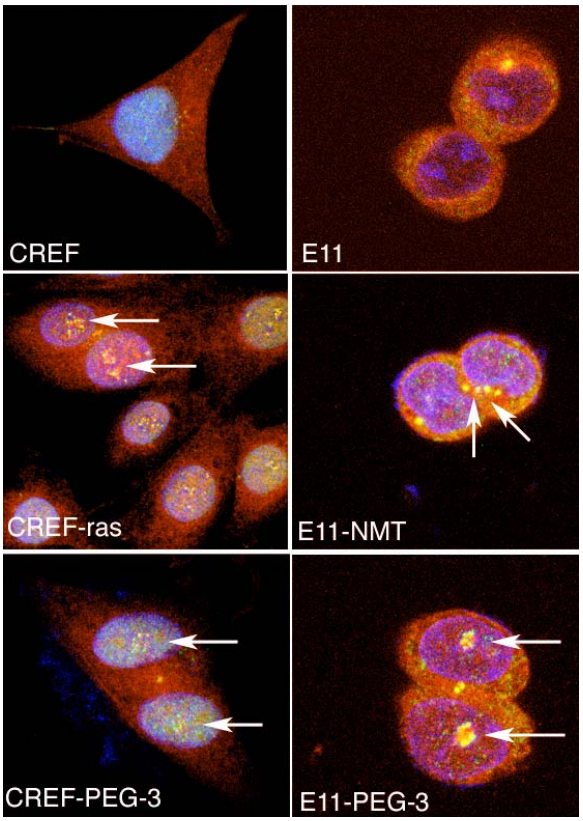

Figure 4. *PEG-3* induces centrosomal amplification. Centrosomes were stained with anti-γ–tubulin and FITC-conjugated IgG (in green). The mitotic spindle was stained with anti–β̃tubulin and rhodamine-conjugated IgG (in red). Nuclei were counterstained with DAPI. Arrows indicate multiple centrosomes. The cell type analyzed is shown in the figure. (From Emdad *et al.,* 121).

respectively, in comparison to CREF cells. The expression level of p21 (waf1, cip1, sdi1, *mda*-6) was almost undetectable in E11-NMT and E11-*PEG-3* cells. As a corollary, the phosphorylated form of NPM (PNPM) could not be detected in CREF and CREF-*PEG-3* cells, but it was induced in CREF-*ras* cells. There was a basal level of PNPM in E11 cells, that was significantly increased in E11-NMT, and E11-*PEG-3* cells. The level of NPM was similar in all the cells. Recent studies have identified the aurora kinase family as another group of proteins involved in regulation of centrosome function, discussed above. Aurora-A is a centrosome-associated kinase and oncogene, over expression of which leads to centrosome amplification, transformation and aneuploidy. The expression of Aurora-A was barely detectable in CREF cells while increased expression was detected in CREF-*PEG-3* and CREF-*ras* cells. The basal expression of Aurora-A was high in E11 cells. However, there was a significant increased expression in E11-NMT and E11-*PEG-3* cells. The expression level of another member of the aurora kinase family (Aurora-B) was unaltered by *PEG-3* indicating the specificity of *PEG-3* in modulating Aurora-A kinase. All these events culminate in centrosomal amplification, a finding observed in *PEG*-3 over expressing cells, suggesting a relationship between expression of this single progression modulating gene and genomic instability.

Based on the novelty and potential importance to cancer progression of rodent *PEG*-3, a genetic element capable of inducing an aggressive phenotype when expressed in rodent and human transformed cells, without displaying transformation potential in normal diploid cells, studies have focused on defining the origin of this gene. These investigations indicate that *PEG*-3 originates from mutation in the rodent growth arrest and DNA damage inducible gene-34 (GADD34) (122, 123). A one-base deletion in rat GADD34 results in a frame-shift and premature appearance of a stop-codon resulting in a C-terminally truncated molecule that is *PEG*-3 (123). This type of mutation in the GADD34 gene is a frequent event during transformation and/or immortalization of rodent cells. Sequencing of the GADD34 gene in a number of independent rat tumor cell lines revealed that in a majority of these the GADD34 gene is mutated to either *PEG*-3 or a *PEG*-3-like gene with similar C-terminal truncations (123). An important function of GADD34 is to inhibit cell growth, predominantly by apoptosis, and we demonstrated that *PEG*-3 or C-terminal truncations of human GADD34 that resemble *PEG*-3 prevent the growth inhibition by both human and rat GADD34. Phosphorylation of p53 by GADD34 is one mechanism by which it inhibits growth and *PEG*-3 could prevent GADD34-induced p53 phosphorylation (123). In contrast, *PEG*-3 was unable to block other GADD34-induced changes, including eIF2α dephosphorylation, indicating that its effects on GADD34 may be related more to its effect on cell growth rather than a global inhibitor of all GADD34 functions. We hypothesize that mutational generation of *PEG*-3 or a similar molecule is a critical event during rodent carcinogenesis. The inherent property of *PEG*-3 to function as a dominant negative of the growth inhibitory property of GADD34 might rescue cells from DNA damage-induced apoptosis leading to growth independence and tumorigenesis (123).

Our data relative to *PEG*-3 supports the hypothesis that either the inhibition of wild-type GADD34 by *PEG*-3 or *PEG*-3-like molecules (when one allele of the GADD34 gene is mutationally inactivated) or facilitation of cell growth by *PEG*-3 itself (either in mono- or bi-allelic inactivation) might be a crucial event in rodent tumorigenesis (123). This raises the interesting question of whether this type of genetic change also occurs in humans. Our preliminary studies aimed at sequencing the human GADD34 gene in a number of human cancer cells revealed no *PEG*-3-like mutations (unpublished data) indicating that unlike rodents, mutational inactivation of the GADD34 gene might not be a frequent event during human carcinogenesis. However, functional inactivation of GADD34 by protein-protein interactions or inactivation of expression by mutation in the promoter region could provide alternate mechanisms for a role of GADD34 in human tumor formation.

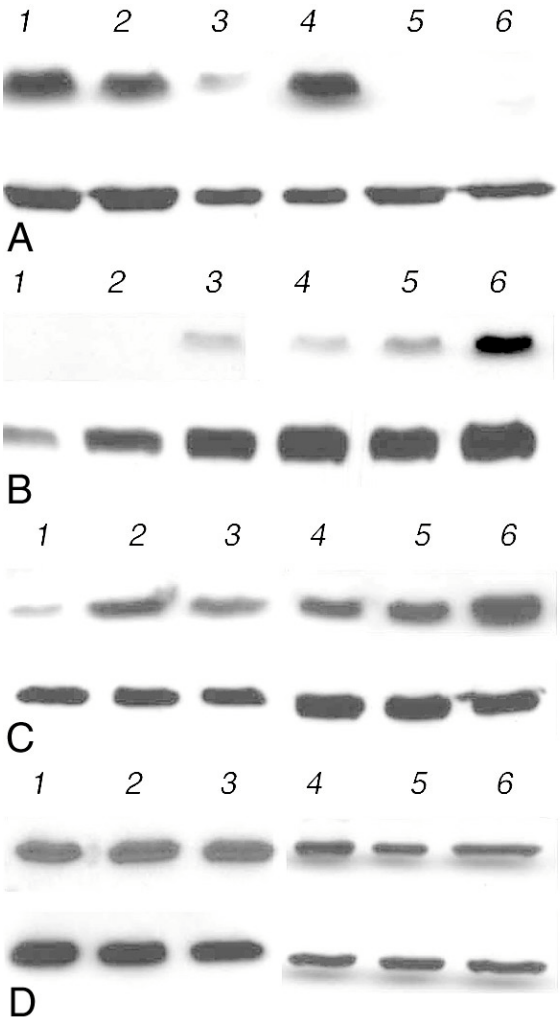

Figure 5. *PEG-3* modulates the expression of specific centrosome-associated proteins. **A.** Western blot analysis showing p21 (waf1, cip1, sdi1, *mda*-6) protein levels in the CREF and E11 series of cell lines. To ensure that equal loading was achieved the same membrane was probed with EF1α. **B.** Western blot analysis of cell lysates from the CREF and E11 series of cell lines probed with an antibody against phospho-T199 nucleophosmin B. As a loading control, the same membrane was probed with an antibody against total nucleophosmin (NPM). **C.** Western blot analysis showing increased Aurora-A kinase protein levels in CREF-*PEG-3*, CREF-*ras*, E11-NMT and E11-*PEG-3* cells in comparison with parental CREF and E11 cells, respectively. The membrane was reprobed with an antibody against EF1α to confirm equal loading. **D.** Western blot analysis of cell lysates from the CREF and E11 series of cell lines probed with an antibody against Aurora-B kinase. As a loading control, the same membrane was probed with an antibody against EF1α. Lane designations: **1**. CREF; **2**. CREF-*PEG-3*; **3**. CREF-*ras*; **4**. E11; **5**. E11-NMT; **6**. E11-*PEG-3*. (From Emdad *et al.,* 121).

11. CONCLUSIONS AND PERSPECTIVES

A priori, one could argue that centrosome anomalies prompt errors during cytokinesis, thereby generating a transient tetraploid state. We hypothesize that a tetraploid state and extra copies of centrosomes arise concomitantly, as a consequence of defects in mitotic progression and aborted cytokinesis. According to this interpretation, centrosome anomalies are a by-product, rather than the cause, of tetraploidization. This implies that centrosome numbers may constitute convenient surrogate markers for polyploidy in tumor tissues. Furthermore, centrosome anomalies are expected to favor chromosome mis-segregation during subsequent cell divisions, and this may explain why the tetraploid state in tumor tissues is not stable but generally progresses to an aneuploid state. Thus, although deregulation of centrosome duplication may not constitute a major primary cause for centrosome amplification in tumors, the hypothesis that centrosome anomalies contribute to tumor development remains attractive and worthy of further investigation.

It is evident from the studies of our group that the *PEG-3* gene cloned in our laboratory (118) induces genomic instability by modulating the expression of several genes that have primary roles in centrosomal duplication. Elucidating the signal transduction pathways by which *PEG-3* induces these defined genomic alteration would result in an enhanced understanding of the role of genomic instability in cancer progression. Moreover, the identification of a gene like *PEG*-3 argues that genetic elements exist in transformed cells that can uniquely enhance cancer progression, without being direct acting oncogenes with transforming functions. Further studies designed to identify and characterize such elements, including progression promoting and progression suppressing genes (131-133), in the human genome will provide important insights into the molecular mechanism by which tumor cells evolve and acquire aggressive cancer phenotypes. Moreover, these human progression-regulating genes should provide novel targets offering a unique opportunity to develop effective cancer therapies.

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