Cancer: Cell Structures, Carcinogens and Genomic Instability
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Viral carcinogenesis and genomic instability

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Abstract. Oncogenes encoded by human tumor viruses play integral roles in the viral conquest of the host cell by subverting crucial and relatively non-redundant regulatory circuits that regulate cellular proliferation, differentiation, apoptosis and life span. Human tumor virus oncoproteins can also disrupt pathways that are necessary for the maintenance of the integrity of host cellular genome. Some viral oncoproteins act as powerful mutator genes and their expression dramatically increases the incidence of host cell mutations with every round of cell division. Others subvert cellular safeguard mechanisms intended to eliminate cells that have acquired abnormalities that interfere with normal cell division. Viruses that encode such activities can contribute to initiation as well as progression of human cancers.

Key words: Aneuploidy, centrosomes, cervical cancer, human papillomavirus, tumor suppressor, viral oncogene.

Viruses and cancer

Viruses are obligatory intracellular parasites and hence their life cycles are irrevocably coupled to that of their host cells. Due to the limited coding capacity of viral genomes that is imposed by packaging limits, viruses have developed strategies to target host cellular regulatory structures and reprogram them for their own purposes. The interplay between a virus and its host cell is a fascinating area of study; during co-evolution host cells have developed intricate defense strategies to restrict viral replication, whereas the intruding viruses have evolved to thwart host cellular antiviral defense mechanisms (reviewed in [1]). Non-productive viral infections can arise if a host cell is intrinsically incapable of supporting the viral life cycle or if the viral genome is mutated and rendered replication defective. Under such conditions viral functions are aberrantly expressed but no infectious progeny is produced, which can have perilous consequences for the host cell (reviewed in [2]).

Approximately 20% of all human cancers may have a viral etiology (reviewed in [3]). The concept of viral carcinogenesis was originally derived from studies with animals where infectious entities, many of which were later identified as retroviruses, were shown to cause formation of malignant tumors. In some rare cases, retroviruses can contribute to carcinogenesis by insertion-

al mutagenesis, where integration of the provirus causes high-level dysregulated expression of a cellular proto-oncogene or disruption of a tumor suppressor. More frequently, however, retroviruses "pick up" cellular proto-oncogene sequences during their replication cycles. Since this process is generally associated with concomitant deletion of viral coding sequences, many oncogenic retroviruses are intrinsically defective for completing the infectious life cycle, and require normal "helper" viruses for replication. Replication of retroviral genomes involves the viral reverse transcriptase enzyme that lacks proofreading mechanisms, and thus is considerably more error-prone than host chromosome replication. Moreover, the acquired host cell-derived sequences do not contribute to the viral life cycle and are not subject to the same degree of mutational restriction as the viral genome. Hence they will accumulate mutations at a significantly higher rate than the remainder of the retroviral genome. In rare cases, the resulting expression of specific mutated versions of such retrovirally transduced cellular genes can endow the infected host cell with a growth advantage relative to the surrounding uninfected cells, and a tumor may form (reviewed in [4]).

Even though the concept of oncogene activation and transmission by retroviruses has not been clearly documented in human cancers, the recognition that retrovirally transmitted oncogenes represent specifically altered versions of cellular proto-oncogenes had a major impact on our understanding of carcinogenic mechanisms. In human cancers, proto-oncogenes are frequently mutated and activated through cell intrinsic mechanisms, including point mutations, gene amplification, gene fusion, or alterations that lead to increased mRNA or protein stability. Moreover, activating mutations of oncogenes isolated from human cancers are often identical to those originally discovered with retrovirally activated oncogenes [5].

Oncogenes of human tumor viruses are virally encoded genes that play integral roles for the viral life cycle. To fulfill their roles in the viral life cycle, human tumor virus oncogenes target critical cellular regulatory circuits, including cellular proto-oncogenes and tumor suppressor pathways, and cause their activation or inactivation, respectively.

Some viral oncogenes also subvert cellular processes that are necessary for maintaining genomic integrity of the host cell. Hence, some human tumor viruses also contribute to human carcinogenesis by creating a cellular milieu that is conducive for the generation and accumulation of activating mutations of cellular oncogenes and/or inactivating mutations of tumor suppressors in the host genome. Such viruses contribute not only to initiation but also to progression of human cancer.

Human papillomaviruses and carcinogenesis

Papillomaviruses are ubiquitous non-enveloped viruses with small 8-kb double-stranded DNA genomes. Only one of the DNA strands is actively tran-

scribed, and the complementary strand does not contain any coding information. The papillomavirus genome can be divided into three parts. The early coding region encodes approximately seven open reading frames (ORFs). Individual early ORFs are denoted by the letter "E" and a number according to their relative molecular size. The lower the number, the longer the corresponding ORF. The late coding region consists of two "L" ORFs, which encode the viral capsid proteins. The non-coding region contains multiple *cis* regulatory elements that modulate viral transcription and genome replication (Fig. 1A) (reviewed in [6]). Approximately 200 different human papillo-

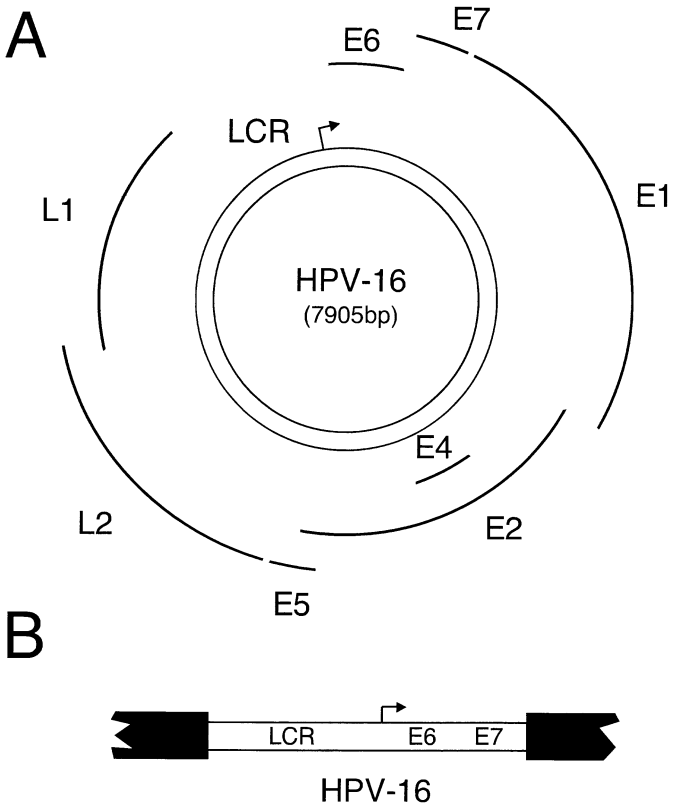

Figure 1. (A) Schematic depiction of the double-stranded circular genome of high-risk HPV-16. Only one of the two DNA strands is actively transcribed and contains all the coding information. The different early (E) and late (L) open reading frames are encoded using each of the different possible phases of translation as indicated by the concentric arcs. The long control region (LCR) does not contain extensive coding potential but contains various *cis* elements that are necessary for the regulation of viral transcription and replication. The position of the major early promoter within the LCR is indicated by an arrow. See text for details. (B) Representation of integrated HPV LCR/E6/E7 sequences in cervical cancer lines. The HPV genes are expressed from the viral promoter within the LCR (indicated by an arrow) expression is dysregulated due to transcriptional and non-transcriptional mechanisms. See text for details.

maviruses (HPVs) have been identified, and additional types likely exist (reviewed in [7]). HPVs display a pronounced tropism for squamous epithelial cells, and approximately 30 HPVs specifically infect mucosal epithelia. The mucosal associated HPVs are classified as "high-risk" and "low-risk" according to the propensity for malignant progression of the lesions that they cause. Low-risk HPVs cause benign warts, which have an extremely low risk for malignant progression. In contrast, infections with high-risk HPVs account for more than 99% of all cervical carcinoma. Worldwide, in excess of 470,000 cervical cancer cases are newly diagnosed each year, and cervical cancer remains a leading cause of cancer death in young women. Since no HPV-specific therapies exist, there are very limited regimens for treatment of late stage invasive cervical cancer, and the death rate has remained unacceptably high at approximately 30% (reviewed in [8]). Cervical cancer incidence is much lower in countries where there is broad access to preventive cytology-based screening programs that allow for detection of potentially pre-cancerous high-risk HPV-associated squamous intraepithelial lesions (SILs). In the US cervical carcinoma accounts for approximately 6% of all cancer cases (13500 per year), and remains frequent in medically underserved segments of the populations. Approximately 20% of human oral cancers, particularly oropharyngeal carcinomas, are also high-risk HPV positive [9]. A fraction of other anogenital tract malignancies such as penile cancer in males and vulvovaginal cancers in females (reviewed in [10]), as well as anal carcinomas that frequently occur in AIDS patients (reviewed in [11]), are also associated with high-risk HPV infections. Even though preventive vaccination strategies using recombinant empty capsid particles yielded promising results [12], it will be decades before they might have a major impact on the incidence of HPV-associated disease (reviewed in [13]).

Due to the small size of their genomes, HPVs do not encode key, rate-limiting replication enzymes, and thus these viruses have adopted a parasitic replication strategy to exploit the cellular DNA replication machinery. Establishing and maintaining a cellular environment conducive for viral genome synthesis is paramount since the HPV life cycle is tightly linked to the differentiation status of the infected keratinocyte. The squamous epithelium is a multilayered organ and only the basal layer contains undifferentiated, actively dividing cells. These cells are the initial targets for infection, and HPVs gain access to these cells either through an injury or at the squamocolumnar transformation zone where basal-like epithelial cells are more readily accessible. Expression of late genes and production of viral capsids, however, only occurs in differentiated epithelial cells. In a normal squamous epithelium, cellular differentiation and proliferation are tightly coupled processes, and cells terminally withdraw from the cell division cycle when they undergo differentiation. To allow for viral genome replication in these growth-arrested cells, HPVs encode regulatory proteins that can uncouple these processes (reviewed in [6]). Consistent with this notion, high-risk HPV E6 and E7 proteins functionally compromise the p53 and retinoblastoma

(pRB) tumor suppressors, respectively [14, 15]. In addition, high-risk HPV E6 can activate transcription of hTERT, the catalytic protein subunit of human telomerase [16].

Infection of the anogenital tract with high-risk HPVs is through sexual contact and is quite frequent in the sexually active population [17]. Most infections with high-risk HPVs are transient and do not cause any clinical symptoms. Persistent high-risk HPV infections, however, can cause potentially pre-malignant SILs. Malignant progression of such lesions is an overall rare event that can take decades to occur (reviewed in [18]). An important hallmark of malignant progression of lesions caused by high-risk HPV infection is the frequent integration of the viral sequences into the host cellular genome. HPV genome integration does not cause insertional mutagenesis, and even though HPVs frequently integrate near common chromosomal fragile sites [19], there are no specific integration HPV sites in the human genome [20]. Integration disrupts the integrity of the viral genome and causes a cellular growth advantage due to increased mRNA stability and expression levels of the remaining viral transcripts [21]. Only the HPV E6 and E7 genes remain consistently expressed in HPV-positive cervical cancers (Fig. 1B). HPV E6 and E7 encode small proteins of approximately 100 and 160 amino acid residues, respectively. They lack enzymatic or specific DNA binding activities and appear to function by associating with cellular protein complexes, thereby subverting their biological functions (reviewed in [22]). Ectopic expression of high-risk HPV E6 and E7 in primary human epithelial cells causes life span extension and permits immortalization [23, 24]. When grown under organotypic conditions, HPV E6/E7-expressing keratinocytes form structures that are reminiscent of high-grade pre-cancerous lesions [25]. Moreover, when transgenic mice with expression of HPV-16 E6 and E7 targeted to basal epithelial cells are exposed to continuous low doses of estrogen, they develop cervical cancers that mirror the human disease [26]. Sustained expression of E6 and E7 is necessary for maintenance of the transformed phenotype of human cervical cancer-derived cell lines even after they have been in culture for decades and have accumulated a plethora of genomic alterations (reviewed in [6]).

Since the HPV genome suffers irreversible physical disruption as a consequence of integration into a host chromosome, carcinogenic progression of a high-risk HPV-infected cell is a terminal event and not part of the normal viral life cycle. Rather, malignant progression ensues as a result of dysregulated expression of HPV E6/E7 genes, which normally play essential roles for the viral life cycle (reviewed in [22]).

Remarkably, the majority of human solid tumors have abnormalities in the pRB and p53 tumor suppressor pathways and maintain stable telomere length (reviewed in [27]), illustrating the notion that the signal transduction pathways targeted by high-risk HPV E6 and E7 oncoproteins are also rendered dysfunctional in many other human solid tumors that are not HPV associated.

HPVs, genomic instability and malignant progression

Genomic instability is a defining characteristic of human solid tumors, and human cancer has been described as a disease of genomic instability [28]. There is much debate as to whether genomic destabilization mechanistically contributes to malignant progression, or if it arises as a consequence of cell cycle checkpoint abnormalities and/or continued division of cells containing chromosomes with eroded telomeres. The mutation rate of normal human cells is exceedingly low, and thus may not suffice to permit accumulation of the multitude of genetic alterations that are necessary for multistep human carcinogenesis (reviewed in [29]). Moreover, human cancer-like cells can be generated *in vitro* by targeting a minimal set of critical regulatory pathways including the pRB and p53 tumor suppressors, protein phosphatase 2A, and telomerase, all of which are also commonly rendered abnormal in human cancers (reviewed in [30]). Such *in vitro* generated human tumor-like cells retain a high degree of genomic stability [31]. This result lends powerful support to the notion that genomic stability does not inevitably ensue in cells with abnormal patterns of proliferation. Genomic instability may require additional alterations or a combination of molecular changes to enable an emerging tumor cell to accumulate the necessary oncogenic mutations, and hence may represent a vital step for cancer formation *in vivo* [32].

Multiple cooperating mechanisms likely contribute to genomic destabilization in tumors (reviewed in [33]). Subversion of "quality control" functions such as cell cycle checkpoints and DNA repair functions allow cells that have accumulated mutations to remain in the proliferative pool. Other oncogenic hits, however, may directly affect genomic instability by generating a mutator phenotype, which enhances the mutation rate at every round of DNA synthesis and cell division of an emerging tumor cell [34, 35].

Cervical cancers exhibit both structural and numerical chromosomal aberrations and genomic instability is observed in early pre-malignant lesions (reviewed in [36]). In addition, ectopic expression of high-risk HPV E6 and E7 in primary human cells can each interfere with genomic stability. In experiments where cells were selected for acquiring resistance to the drug *N*-phosphonoacetyl-L-aspartate (PALA), HPV E6-expressing cells accumulated structural chromosomal abnormalities, whereas numerical chromosomal abnormalities and aneuploidy emerged in HPV E7-expressing cells [37].

Tetrasomy/multinucleation

Morphological examination of HPV-associated cervical lesions revealed the presence of distinct nuclear abnormalities, including enlarged nuclei as well as multinucleation (reviewed in [38]). Enlarged nuclei are a hallmark of increased ploidy and numerical chromosomal abnormalities. Lesions caused by high-risk HPV infections, but not those associated with low-risk HPV

infections, showed an increased degree of tetrasomy [39, 40]. Ectopic high-risk HPV E6 or E7 expression can each independently induce tetraploidization both in actively dividing basal and intrinsically growth-arrested suprabasal cells [41, 42]. The mechanism of tetrasomy induction by HPV oncoproteins has not been delineated. Expression of high-risk HPV E6 may cause increased ploidy and multinucleation through inactivation of the p53 tumor suppressor [43]. Interestingly, however, the ability of HPV E7 to induce tetrasomy is unrelated to the capacity to inactivate the retinoblastoma tumor suppressor [42].

Tetrasomy is often regarded as a prelude to aneusomy, since, as discussed in more detail later, such cells are more prone to mitotic errors when they undergo additional rounds of cell division (reviewed in [44]). Tetraploidy arises in cells that undergo DNA synthesis without completing nuclear and cellular division, most frequently as a consequence of cytokinesis problems. It is important to point out that a tetraploid cell will have to be able to successfully complete a subsequent cell division to generate aneuploid progeny. Cells that re-encounter cytokinesis problems, however, will become polyploid and/or multinucleated (reviewed in [44]). The emergence of cells with severe nuclear abnormalities may be of relevance diagnostically, but since such cells were generated through persistent cytokinesis defects, and thus are incapable of undergoing full cell division, they represent abortive structures [48, 49] that do not contribute to malignant progression.

Centrosome duplication errors, multipolar mitoses and aneusomy

Aneuploidy arises as a consequence of chromosome segregation errors during mitosis. One of the typical mitotic abnormalities that pathologists have observed in high-risk HPV-associated pre-malignant lesions and cancers are tri-polar mitotic figures (Fig. 2) [45]. Such abnormalities can arise when cells contain supernumerary mitotic spindle pole bodies, centrosomes. Centrosomes consist of two centrioles that are surrounded by a pericentriolar protein matrix, which functions to anchor microtubules during mitosis. Immediately after cell division each daughter cell contains a single centrosome, which undergoes semi-conservative duplication in exact synchrony with the cell division cycle. Entry into S phase of the cell division cycle is believed to generate a "licensing signal" that renders the centrosome competent for duplication. Once a centrosome is licensed for duplication the two centrioles separate and each serves as a template for synthesis of a single daughter centriole. Daughter centriole synthesis is complete at the end of S phase, and the resulting two centrosomes form the mitotic spindle pole bodies that are critical for bipolar mitosis (Fig. 3). The mechanistic details of this unique and important cellular process remain enigmatic (reviewed in [46]).

Abnormal centrosome numbers can arise by two principal mechanisms. As described in the previous section, a cell that experiences cytokinesis problems

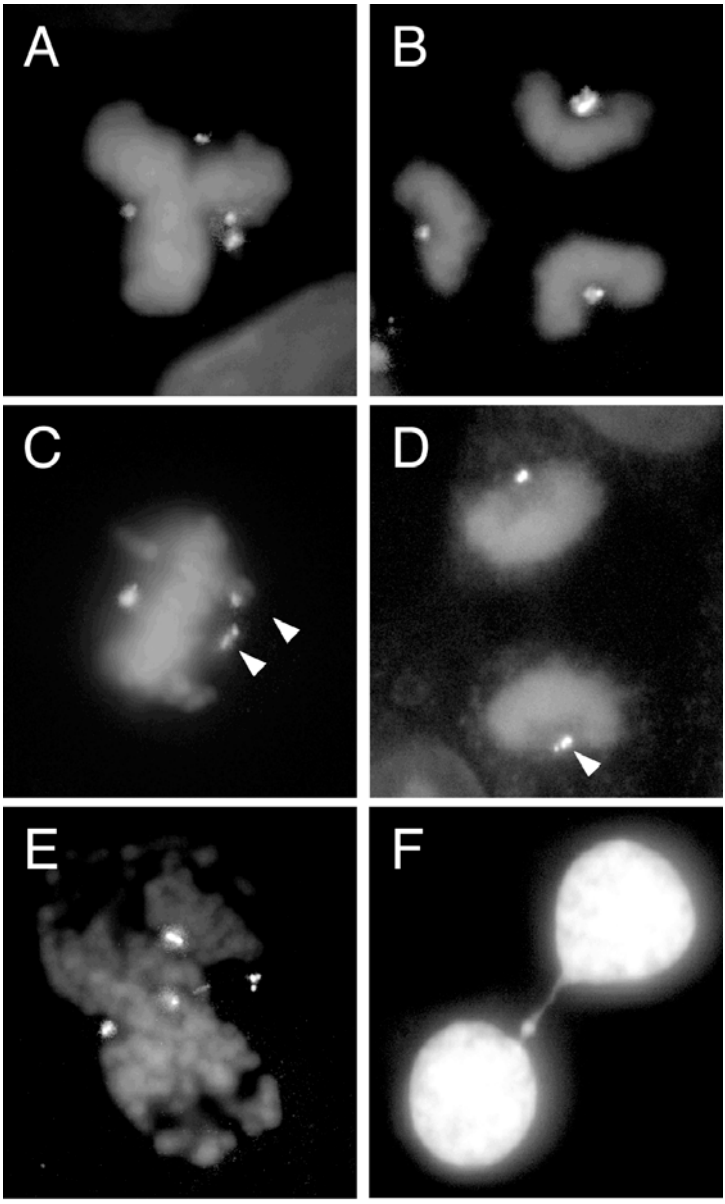

Figure 2. Examples of mitotic abnormalities in HPV-16 oncogene-expressing cells. HPV-16 E7 oncogene expression causes centrosome duplication errors, which can give rise to tripolar metaphases (A), which can undergo anaphase progression (B). Multiple centrosomes (indicated by arrowheads) in HPV-16 E7-expressing cells can undergo coalescence and form abnormal bipolar metaphases (C) and anaphases (D). Highly irregular multipolar metaphase in HPV-16 E7-expressing cells (E). HPV oncogene-expressing cells also contain centrosome-independent mitotic abnormalities including anaphase bridges (F). These may be caused by dicentric chromosomes that might have formed as a consequence of breakage fusion bridge cycles. See text for details.

progresses into a G_1-like phase without completing cellular and/or nuclear division (Fig. 3). Hence, the resulting tetraploid and/or binucleated cell contains two centrosomes. If such a cell re-enters the cell division cycle, it will also duplicate its centrosomes and enter mitosis with four centrosomes. Each individual centrosome may then act as a mitotic spindle pole body and a tetrapolar mitotic spindle can form. Some centrosomes may not fully separate and multiple centrosomes may form a single mitotic spindle pole leading to tripolar or bipolar mitosis (Fig. 2). If such cells can complete cell division and do not re-encounter cytokinesis problems (which caused the formation of the tetraploid cell during the previous round of the cell division cycle), there is an increased probability for chromosome missegregation, leading to formation of aneuploid daughter cells (reviewed in [47]). In this scenario, centrosome abnormalities are generated through normal duplication cycles and arise as a consequence of cytokinesis defects and not through aberrant synthesis. In many cases, however, including in cells lacking p53 tumor suppressor function, cytokinesis errors persist [48]. Such cells may be able to reduplicate their chromosomes and centrosomes but remain incapable of successfully undergoing cell division, and acquire progressive nuclear abnormalities including large multilobulated nuclei, multiple nuclei or micronuclei (Fig. 3). Such abnormal cells are ultimately removed form the proliferative pool by apoptosis, senescence or other abortive mechanisms, and hence are unlikely to contribute to carcinogenic progression [48, 49].

Centrosome abnormalities, however, may also emerge as a primary defect in cells through uncoupling of centrosome duplication from the cell division cycle. In such a scenario, a single maternal centriole may serve as a template for the synthesis of more than one daughter centrioles during S phase, or alternatively newly formed daughters may be immediately "licensed" to serve as templates for the synthesis of granddaughters (Fig. 3). In the late 19th century, the eminent German embryologist Theodor Boveri performed studies with polyspermic embryos and recognized that abnormal centrosome numbers gave rise to abnormal multipolar mitoses [50, 51], which severely compromised the viability of the resulting embryos. Based on earlier observations that human cancer cells frequently displayed abnormal multipolar mitotic figures [52], he first postulated that "a single multipolar mitosis going on in a healthy tissue, caused perhaps by the simultaneous multiple division of the centrosome, might produce the primordial cell of a malignant tumor" [53]. This attractive hypothesis, however, remains largely unproven experimentally.

Like many other human tumors, cervical cancers as well as high-risk HPV-associated pre-malignant lesions contain centrosome abnormalities [49], and their incidence appears to increase in parallel with malignant progression [54]. In addition to numerical aberrations, high-risk HPV-associated lesions also display structural centrosome abnormalities, including excess pericentriolar material [49], but the mechanistic basis of the structural defects has not been assessed in detail. Cervical cancer is unique among human solid tumors in that the fundamental carcinogenic insult that causes these tumors, infection with

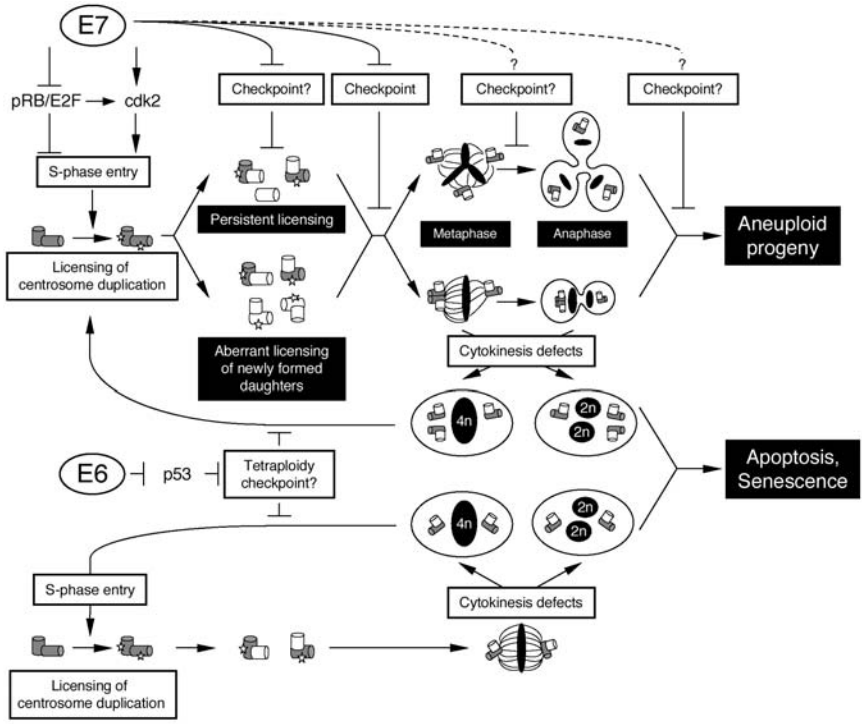

Figure 3. Centrosome abnormalities in human tumors can arise by different mechanisms. HPV-16 E7 oncoprotein expression in primary human cells induces centrosome duplication errors that can lead to mitotic abnormalities, chromosome missegregation and aneuploidy. The retinoblastoma tumor suppressor restricts DNA replication in normal human cells by forming a transcriptional repressor complex with members of the E2F transcription factor family. HPV E7 associates with pRB and induces its proteolytic degradation. E2F transcription factors now act as activators of gene expression. Cyclin E is a transcriptional target of E2F and results in increased cdk2 activity. Initiation of DNA synthesis also generates a "licensing signal" that renders each of the centrioles competent for duplication. Normal centrosome duplication is coupled to S-phase progression, and each maternal centriole serves as a template for a single daughter. In addition to inducing aberrant S-phase progression, HPV-16 E7 expression uncouples centrosome duplication from the cell division cycle, either by retaining the licensed state of the maternal centriole ("persistent licensing"), which causes the formation of multiple daughters from a single maternal template, or the newly synthesized daughters in E7-expressing cells may be immediately licensed for duplication, causing formation of granddaughters [102]. Cells that acquired supernumerary centrosomes will either enter a multipolar metaphase or an abnormal bipolar metaphase when individual centrosomes coalesce and form a single mitotic spindle pole body. Abnormal metaphase to anaphase progression may be restricted through a checkpoint, as there is an eight- to tenfold difference between abnormal metaphases and anaphases in HPV-16 E7-expressing cells. If such abnormal cells can complete nuclear and cellular division, aneuploid progeny may be formed. There may be an additional checkpoint that constrains cell division of abnormal mitotic cells, as it has been shown that some tumors remain largely diploid despite the presence of excessive numerical centrosome abnormalities and related mitotic abnormalities [103]. Cells that encounter cytokinesis defects may decondense their chromosomes and reenter a tetraploid G_1-like state. Alternatively cells may complete nuclear, but not cellular, division and become binucleated. Tetraploid cells may reenter the cell division cycle causing additional reduplication of centrosomes. Centrosome abnormalities in HPV-16 E6-expressing cells do not arise in diploid cells, but accumulate in parallel ...
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high-risk HPVs, is known at a molecular level (reviewed in [22]). Hence it affords the opportunity to determine whether ectopic expression of HPV oncogenes in normal human cells could induce centrosome abnormalities. Populations of primary human epithelial cells with stable expression of high-risk HPV genomes or HPV E6 or E7 each showed an increased incidence of numerical centrosome abnormalities [49, 55], and up to 30% of all mitoses in cells co-expressing E6 and E7 showed evidence of centrosome-associated mitotic abnormalities. Most notably, expression of low-risk HPV-encoded E6 and/or E7 expression did not affect centrosome homeostasis [49].

Since the high-risk HPV E6 and E7 oncoproteins target distinct albeit cooperating oncogenic cellular pathways, the finding that cells expressing HPV E6 or E7 each developed centrosome abnormalities was initially somewhat perplexing. More careful analysis of E7-expressing keratinocytes revealed that centrosome abnormalities were detected in mononuclear, diploid cells. In contrast, centrosome abnormalities in HPV E6-expressing cells were mostly confined to cells with overt nuclear abnormalities such as multinucleation or enlarged, multilobulated nuclei. Hence, centrosome abnormalities in high-risk HPV E6- and E7-expressing cells arise by different mechanisms. In E6-expressing cells, centrosomes accumulate as a consequence of persistent cytokinesis defects that are most likely caused by subversion of p53 tumor suppressor function. Since such abnormal cells often expressed markers of cellular senescence, they are unlikely to remain in the proliferative pool, and may not give rise to viable daughters. In contrast, HPV E7 expression triggers centrosome abnormalities in normal diploid cells [56].

Consistent with Boveri's hypothesis that oncogenic insults may trigger centrosome abnormalities by "the simultaneous multiple division of the centrosome", transient expression of HPV-16 E7 was shown to rapidly cause centrosome abnormalities in normal cells within one or two cell division cycles. Moreover, expression of HPV-16 E7 in a U2OS human osteosarcoma cell line with stable expression of GFP-centrin that marks individual centrioles, demonstrated that E7 expression is sufficient to induce aberrant centriole synthesis. As expected, expression of the HPV E6 oncoprotein did not cause similar defects [56].

Diploid human cells with overduplicated centrosomes may undergo multipolar mitoses (Figs 2, 3), but it is difficult to envision how daughter cells generated by multipolar mitosis from a diploid cell may actually gain chromosomal material rather than losing chromosomes and becoming hypodiploid. As

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Figure 3. (Continued from previous page) ... with nuclear abnormalities. This may be related to the ability of HPV E6 to inactivate the p53 tumor suppressor that is a component of mitotic checkpoint control, and p53-deficient cells have been shown to develop centrosome and nuclear abnormalities as a consequence of cytokinesis failure [48]. Cells with persistent cytokinesis defects will accumulate progressive nuclear and centrosome abnormalities, but since they are defective for completing cell division, they are unlikely to undergo clonal expansion and contribute to carcinogenic progression. Such cells may be of diagnostic significance but they represent abortive structures that are eventually removed from the replicative pool [48, 49]. See text for detail.

mentioned previously, cells with abnormal centrosome numbers are not necessarily destined to undergo multipolar cell division. Indeed, we observed an approximately tenfold difference in the incidence of multipolar metaphases *versus* multipolar anaphases, suggesting that there may in fact be control mechanisms that thwart progression of multipolar mitotic processes in diploid cells (Fig. 3) [56]. As mentioned previously, multiple centrosomes can form a single mitotic spindle pole body through centrosome coalescence (reviewed in [57]). Under such conditions, a diploid cell may undergo bipolar, albeit potentially asymmetric, cell division with abnormal chromosome segregation (Fig. 2). In such a scenario, one of the resulting daughters may gain chromosomal material and become aneuploid (Fig. 3). Centrosome coalescence and associated mitotic abnormalities have indeed been observed in HPV oncogene-expressing cells [58].

Boveri's prediction that centrosome duplication errors in normal cells may contribute to carcinogenesis [53] could not yet be proven in this system; however, recent studies with transgenic mice that express HPV-16 E6 or E7 separately yielded results that are at the very least consistent with his hypothesis. Mice engineered to express HPV-16 E7 in basal epithelial cells developed high-grade cervical dysplasia that progressed to frank cervical carcinomas. In contrast, HPV-16 E6-expressing mice only developed low-grade cervical dysplasia, which failed to undergo malignant progression [59]. Not surprisingly, a similar fraction of cells exhibited centrosome abnormalities in lesions of HPV E6- or E7-expressing animals [59]. Hence, detection of centrosome abnormalities in a tumor *per se* cannot be used as a generic predictor of carcinogenic progression, but the finding that transgenic HPV-16 E7-expressing animals develop tumors is consistent with Boveri's model that aberrant centrosome duplication may contribute to tumorigenesis. In contrast, centrosome abnormalities that occur in cells with nuclear abnormalities may represent abortive events triggered by persistent cytokinesis defects.

Even though pRB inactivation can give rise to mitotic abnormalities and cytokinesis problems due to mitotic checkpoint abnormalities [60], expression of HPV-16 E7 can induce centrosome duplication errors in cells that lack pRB as well as the related pocket proteins p107 and p130 [61]. Hence, this ability of HPV-16 E7 is at least in part independent of the ability to target pRb and/or p107 and p130. Strikingly, however, inhibition of cdk2 activity in E7-expressing cells abrogates the ability of E7 to induce aberrant centrosome synthesis, whereas it does not similarly affect normal centrosome duplication. Treatment of E7-expressing cells with the small molecule cdk2 inhibitor indirubin-3'-monoxime dramatically decreased the steady level of centrosome abnormalities in E7-expressing cells, and strikingly reduced the degree of aneuploidy in such cells [62]. Hence, whereas cdk2 activity may not be strictly necessary for cell division and centrosome duplication [63, 64], aberrant cdk2 activity may cause aberrant centriole synthesis and centrosome abnormalities [62].

Other mitotic abnormalities in HPV oncogene-expressing cells

Examination of mitotic structures in HPV-16 E6- and/or E7-expressing cells also revealed evidence for mitotic abnormalities in cells with normal centrosome numbers. A fraction of the bipolar mitoses showed evidence for lagging chromosomal material [65]. The mechanistic basis has not been investigated in detail, and it is not clear whether the observed material represents entire chromosomes or sub-chromosomal fragments. Nevertheless, such unattached DNA structures may be aberrantly segregated during cell division and could conceivably contribute to aneuploidization.

Examination of anaphase cells revealed an increased incidence of anaphase bridges (Fig. 2), indicative of dicentric chromosomes that may have been generated by breakage fusion bridge (BFB) cycles [66]. Chromosome fusions can occur when telomeres are eroded [67], or when mitosis proceeds in the presence of double-strand DNA breaks. Anaphase bridges were observed in early passage primary cells that possess long telomeres [65]. Hence, dicentric chromosomes in HPV-16 E7-expressing cells are likely caused by double-strand DNA breaks. Indeed, staining of HPV E7 oncoprotein-expressing cells with an antibody specific for phosphorylated histone H2AX (γ-H2AX) revealed the presence of distinct foci that are indicative of double-strand DNA break repair [65]. It is not clear whether E7 induces double-strand DNA breaks, whether it inhibits DNA break repair, or if it somehow stabilizes γ-H2AX-positive chromatin structures. The presence of double-strand DNA breaks in E7-expressing cells could provide for a mechanistic rationalization of the observation that HPV-16-expressing cells have a higher propensity for integration of plasmid DNA [68]. This suggests that integration of the HPV genome into a host chromosome, which frequently occurs during malignant progression (Fig. 1B), might be triggered by expression of the high-risk HPV E7 oncoprotein. The HPV-16 E6 oncoprotein may interfere with single-strand DNA break repair by interacting with the repair protein XRCC1 [69].

High-risk HPV E6 and E7 proteins can subvert the functions of multiple mitotic cell cycle checkpoints [70, 71]. This may be related to the ability of HPV E6 to target the p53 tumor suppressor protein. HPV E7-induced pRB destabilization may also be significant, since loss of pRB function compromises the accuracy of mitosis by causing aberrant expression of the mitotic cell cycle checkpoint protein mad2 through an E2F-dependent pathway [60]. Transcriptional profiling analyses have confirmed dysregulation of mitotic functions in cervical cancer and high-risk HPV-expressing cell lines [72, 73].

Genomic instability induced by other viruses

Infections with human tumor viruses other than high-risk HPVs also cause specific aberrations of cellular and nuclear morphology. The human T cell leukemia virus HTLV-1 causes adult T cell leukemia/lymphomas (ATL),

which are characterized by the appearance of cells containing characteristic flower-shaped multilobulated nuclei. The nuclear abnormalities of these "flower cells" are likely caused by HTLV-1 infection. Comparative genomic hybridization analysis showed evidence for complex and dynamic aneuploidy, particularly in highly aggressive ATL [74, 75]. The HTLV-1 tax oncogene can interact with the mitotic checkpoint protein mad1 [76], and thereby subverts mitotic checkpoint control. In addition, HTLV-Tax can interfere with cellular DNA repair by forming a complex with the chk2 checkpoint kinase [77]. Indeed Tax accumulates in discreet nuclear foci, the "Tax speckled structures" (TSS) [78], which also contain chk2 and the DNA damage response factor 53BP1 [77]. The Tax protein can also form a complex with the chk1 protein [79]. Hence, similar to HPV oncogenes, HTLV-1 Tax can interfere with DNA damage repair as well as chromosomal segregation (reviewed in [80]), and Tax expression in primary human cells causes the emergence of numeric as well as structural genomic alterations [81].

Expression of the HIV-1 vpr gene was reported to cause multipolar mitotic spindle formation, centrosome abnormalities and chromosome breaks that lead to gene amplification and micronuclei formation in some cells [82–84].

Epstein-Barr Virus (EBV) is the only known human member of the γ-1 herpesvirus (lymphocryptovirus) family with a large 184-kb double-stranded DNA genome. EBV infections are very common in the human population and cause infectious mononucleosis. EBV infections can also contribute to B and T cell lymphomas, oropharyngeal carcinomas, gastric carcinomas and potentially other human tumors, often after a lengthy latency period. Immunosuppression as well as accumulation of cellular mutations may contribute to carcinogenic progression. EBV encodes a number of genes that have oncogenic activities in tissue culture systems (reviewed in [85]). It has recently been reported that expression of the EBV oncoprotein latent membrane protein 1 (LMP-1) in human epithelial cells inhibits DNA repair processes and induces micronucleus formation. This suggests that expression of some EBV oncoproteins may affect host genomic stability, thereby facilitating malignant progression [86].

Kaposi's sarcoma-associated herpesvirus (KSHV) alias human herpesvirus 8 (HHV 8) is a recently described member of the γ-2 herpesvirus (rhadinovirus) family [87]. KSHV infections cause Kaposi's sarcoma, some forms of multicentric Castleman's disease, as well as other B cell proliferative diseases including body cavity-based and primary effusion lymphomas (reviewed in [88]). KSHV encodes a viral D-type cyclin, cyclin K, that induces hyperproliferation through subversion of the retinoblastoma tumor suppressor pathway. In the absence of p53 function, cyclin K-expressing cells can undergo multiple rounds of S phase (Fig. 3). Due to cytokinesis defects, cells become polyploid and accumulate supernumerary centrosomes, which can result in aneuploidy [89, 90]. Infection of primary human umbilical vein endothelial cells with KSHV causes marked centrosome-associated mitotic abnormalities, misaligned and lagging chromosomes, anaphase bridges and pronounced nuclear abnormalities [91], suggesting that some KSHV proteins can interfere with genomic integrity.

The large tumor antigen (TAg) of SV40 forms a complex with the Nijmegen breakage syndrome protein (NBS1) that plays a key role in modulating double-strand DNA break repair. This leads to aberrant replication of cellular and viral genomes, resulting in polyploidy and increased SV40 genome copy numbers in infected cells [92]. In addition, SV40 TAg associates with bub-1 and bub-3 mitotic checkpoint proteins, thereby disturbing mitotic fidelity [93]. Ectopic expression of large tumor antigens encoded by some JC human polyomavirus strains were also reported to trigger numeric and structural chromosome aberrations [94].

Hepatitis B virus is a human member of the hepadnaviridae family that causes hepatitis. Chronic hepatitis can progress to cirrhosis and ultimately to hepatocellular carcinoma. Progression is a slow process that often takes several decades to occur [95]. BV-associated liver cancers are genomically unstable and the HBV X protein (Hbx) can induce supernumerary centrosomes and multipolar spindles that are associated with defective mitoses and abnormal chromosome segregation as well as formation of multinucleated cells and micronuclei [96, 97]. Treatment of Hbx-expressing cells with antagonists of the Ran GTPase interacting nuclear export receptor Crm1 [96] or an inhibitor of mitogen-activated protein/extracellular signal-regulated kinase (MEK) 1/2, reduced the incidence of centrosome abnormalities [97]. Interestingly, the adenovirus E1A oncoprotein was also reported to induce centrosome abnormalities through a pathway that depends on the integrity of Ran-dependent nucleocytoplasmic transport [98].

Hence, similar to high-risk HPVs, a number of other human tumor viruses may not only contribute to initiation of tumorigenesis by targeting cellular control mechanisms such as the retinoblastoma or p53 tumor suppressor pathways that would normally restrict proliferation in infected host cells, but also contribute to carcinogenic progression through induction of genomic instability.

Induction of genomic instability and “hit-and-run” carcinogenesis

A credible viral etiology of a given human cancer should conform to Koch’s postulates or some more recent incarnation of these criteria (reviewed in [99]). The “hit-and-run” model of carcinogenesis is based on the hypothesis that an infectious agent may contribute to carcinogenesis by providing only a temporary oncogenic insult that is not required to persist during later stages of carcinogenic progression (reviewed in [100]). As a result, the infectious agent may no longer be detected in an active form in all cells of a cancer. One rationalization of this model may include infectious agents whose sole biological activity is to destabilize the host genome but, unlike the high-risk HPVs, do not directly target other cellular regulatory pathways. In such a scenario the mutagenic stimulus may contribute to initiation of carcinogenesis by providing the necessary genomic mutability that allows for inactivation of cellular signal transduction pathways that normally restrict cellular proliferation or induce a trophic

sentinel response in aberrantly proliferating cells. Abrogation of cell cycle checkpoints, particularly those that ensure mitotic fidelity, may be necessary for perpetuation of genomic instability, and hence it may be argued that at later stages of carcinogenic progression the mutator activity provided by such infectious entities may no longer be necessary. Since excessive genomic instability ("error catastrophe", [101]) may ensue when the initiating mutator activity is retained there may in fact be a powerful evolutionary advantage for such a stimulus to be removed. Although attractive, such a model will be very difficult to prove in experimental models or through epidemiological studies.

Concluding remarks

Studies with DNA tumor virus oncogenes have been instrumental in the discovery of critical growth regulatory pathways that control proliferation, apoptosis and differentiation in normal human cells. Based in part on these discoveries it has been possible to define a minimal set of regulatory nodes that are rendered dysfunctional in almost any human solid tumor [27]. Tumorigenic human cell populations can be generated when these pathways are disrupted *in vitro* (reviewed in [30]). Unlike naturally occurring human tumors such artificially generated human tumor-like cells do not exhibit marked genomic instability [31]. Thus, it appears that genome destabilization may be a necessary step to set the stage for carcinogenic progression. It is an exciting possibility that the study of viral oncoproteins will once again be instrumental in discovering cellular pathways that control the genomic integrity, and that these studies will have important ramifications for our understanding of tumorigenic pathways. Cellular processes that control genomic instability may also be attractive targets for development of novel anticancer therapies. Inhibition of genomic instability in early pre-malignant lesions may restrain malignant progression, whereas therapeutic interventions that lead to increased genomic destabilization in later stage tumors may create genomic chaos [101] that could interfere with clonal expansion and the viability of the tumor [29].

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