Overexpression of Eg5 Causes Genomic Instability and Tumor Formation in Mice

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Abstract

Proper chromosome segregation in eukaryotes is driven by a complex superstructure called the mitotic spindle. Assembly, maintenance, and function of the spindle depend on centrosome migration, organization of microtubule arrays, and force generation by microtubule motors. Spindle pole migration and elongation are controlled by the unique balance of forces generated by antagonistic molecular motors that act upon microtubules of the mitotic spindle. Defects in components of this complex structure have been shown to lead to chromosome missegregation and genomic instability. Here, we show that overexpression of Eg5, a member of the Bim-C class of kinesin-related proteins, leads to disruption of normal spindle development, as we observe both monopolar and multipolar spindles in Eg5 transgenic mice. Our findings show that perturbation of the mitotic spindle leads to chromosomal missegregation and the accumulation of tetraploid cells. Aging of these mice revealed a higher incidence of tumor formation with a mixed array of tumor types appearing in mice ages 3 to 30 months with the mean age of 20 months. Analysis of the tumors revealed widespread aneuploidy and genetic instability, both hallmarks of nearly all solid tumors. Together with previous findings, our results indicate that Eg5 overexpression disrupts the unique balance of forces associated with normal spindle assembly and function, and thereby leads to the development of spindle defects, genetic instability, and tumors. [Cancer Res 2007;67(21):10138–47]

Introduction

Cell division is a complex process requiring proper segregation of chromosomes to each of the daughter cells during mitosis. This process involves centrosomal duplication and migration, and proper assembly of the mitotic spindle. The early stages of mitotic spindle assembly require the nucleation and cross-linking of cytoplasmic microtubules into polar microtubule bundles radiating outward from the duplicated centrosomes, with minus ends located proximal to the centrosome and plus ends located distally. Arrangement of microtubules allows for the separation and migration of centrosomes to opposing poles of the spindle and the formation of an antiparallel microtubule lattice between them (1, 2). Although many steps have been elucidated in centrosome duplication, little is known about the activities that normally coordinate the process within the cell cycle. Recent studies, however, have shown that defects in spindle assembly can cause genetic dysfunction, including gene amplification and/or chromosomal instability (3, 4).

Genetic instability is a hallmark of virtually all solid tumors (5, 6). An important question that has arisen over the years relating to cancer and chromosomal instability is whether the aneuploid state simply reflects the clonal expansion of a rare aberrant cell, or if rather an overall change in the rate of chromosomal loss, gain, and/or rearrangements is responsible for the generation of a group of “mutagenic” cells (7, 8). The existence of a mutagenic cell population would be consistent with the hypothesis that cancer is a multihit disease and that the accumulation of genetic alterations over time results in cells that evolve toward cancer (8). Recent work has shown that mitotic spindle checkpoints and chromosomal segregation pathways are well conserved in mammalian cells and that mutations in these pathways are capable of producing aneuploid cells as seen in naturally occurring cancers featuring chromosomal instability (9–13).

Previously, we inventoried a series of spontaneous leukemias and lymphomas occurring in mice expressing high levels of murine leukemia viruses (MuLV) for recurrent somatically acquired proviral insertion sites. Genomic regions found to be targeted by MuLV insertions in two or more independent tumors were termed lymphoid viral insertion sites (LVIS) and were numbered sequentially. These common integration sites occur in the vicinity of candidate cancer genes and are thought to induce the oncogenic expression of these genes through the process of insertional mutagenesis (14). Lvisi1 is a common integration site in mouse lymphoid leukemias and lymphomas (15). Two genes are located near Lvisi1-Hex, a homeobox-containing transcription factor, and Eg5, a member of the BimC family of kinesin-related proteins (KRP). The fact that these genes are overexpressed in tumors containing insertions at Lvisi1 suggests that they may contribute to lymphomagenesis (15). Originally identified in Xenopus laevis (16), Eg5 is a plus end–directed KRP that associates with microtubules of the mitotic spindle (17). It is also involved in the microtubule dynamics of postmitotic neurons, with enrichment at the distal regions of developing processes (18). A highly conserved NH2-terminal motor domain places Eg5 in the BimC family of KRP, which includes cut7 from Schizosaccharomyces pombe (19), Kip1p and Cin1p from Saccharomyces cerevisiae (20, 21), KLP61F from Drosophila melanogaster (22), XIeG5 from X. laevis (17), and HseG5 from humans (23). Eg5 functions as an antiparallel homotetrameric structure, having two motor domains at opposing ends of a central stalk (17, 24), and is capable of binding and generating forces upon...
microtubules of the mitotic spindle that can directly contribute to spindle assembly and elongation (25, 26). Current studies show that Eg5 and related BimC family members carry out similar functions, and that mutations in this family of genes cause failure of centrosome separation, spindle assembly, and maintenance of a bipolar spindle (17, 19, 20, 22). Additionally, Eg5 inhibition by immunodepletion, antibody injection, RNA interference, and small-molecule inhibitors, such as monastrol, results in mitotic arrest and monopolar spindles (17, 20, 27, 28).

To determine if aberrant expression of Eg5 could perturb mitotic spindle formation, promote genomic instability, and subsequently lead to cancer development, we generated transgenic mice that overexpress Eg5 and observed them for effects on spindle assembly and cancer development. Our results indicate that overexpression of Eg5 leads to abnormal spindle formation. Additionally, overexpression of Eg5 results in genomic instability and ultimately results in the development of a broad spectrum of tumors.

Materials and Methods

Generation of Pim1-Eg5 transgenic mice. To generate transgenic mice overexpressing Eg5, a 3.3 kb mEg5 cDNA clone was subcloned into the Eag1/XhoI site of the TKO-G90 and a 9.2 kb SalI fragment of TKO-G90-Eg5 was injected into FVB pronuclei and implanted into FVB/Nj recipient mice. The TKO-G90 construct was previously reported to drive expression of cDNAs in hematopoietic tissues (29). The plasmid harboring the final transgene construct (pPim1-Eg5) was sequenced to confirm the integrity of the Eg5 open reading frame. For transgene identification, tail clips from founder animals were incubated overnight at 55°C. The founder mice were identified by Southern blot analysis of KpnI-digested genomic tail DNA, or by PCR analysis using primers specific to Eg5 and observed them for effects on spindle assembly and cancer development. Our results indicate that overexpression of Eg5 leads to abnormal spindle formation. Additionally, overexpression of Eg5 results in genomic instability and ultimately results in the development of a broad spectrum of tumors.

Tissue history. Wild-type and transgenic mice were sacrificed by CO2 asphyxiation, and tissues were dissected and placed into buffered zinc-formalin (Z-fix; Anatech) and embedded in paraffin. Tissue histology was assessed by an experienced pathologist blinded to the genotype of the animals under examination.

Generation of MEF lines. MEFs were derived from 13.5-day-old wild-type and transgenic embryos. After removal of the head, liver, and gastrointestinal tract, each embryo was washed with PBS and minced, and the tissue was placed in a 15-mL conical tube. After centrifugation, tissue pellets were resuspended in 1 mL of trypsin solution (0.25% trypsin, 0.005% EDTA) and the tissue was digested at 37°C for 10 min. After pipetting several times, embryos were further digested with 1 mL of trypsin at 37°C for 10 min. Trypsin was inactivated with DMEM (Invitrogen) containing 10% fetal bovine serum (FBS), 2 mM/L L-glutamine, and 2% penicillin/streptomycin. Following pipetting several times, single-cell suspensions were plated on 100-mm tissue culture dishes and incubated at 37°C for 2 to 3 days until the cells reached confluency. These cultures were designated P0.

Immunohistochemistry. Early-passage MEFs (P2–P4) were plated at 5 × 10^4 cells per 100-mm tissue culture plate and grown to ~70% to 80% confluency. Mitotic shake off was carried out by carefully striking sides of tissue culture dishes and collecting medium containing rounded and less adherent mitotic cells. Collected cells were spun down onto glass slides in a cytosin centrifuge at 500 × g for 5 min. Cells were fixed in 4% paraformaldehyde prepared in 4% polyethylene glycol-8000/PEM buffer [80 mM/L K-PIPES (pH 6.8), 5 mM/L EDTA (pH 7), 2 mM/L MgCl2], rinsed in PBS, blocked in 2% bovine serum albumin, 2% normal goat serum, and 0.5% SDS plus 0.25% Triton X-100, then incubated with primary antibody overnight. Mitotic spindles were stained with a FITC-conjugated β-tubulin (Sigma) and an anti-rabbit cytochrome-3 (Cy3)-conjugated secondary antibody. Centrosomes were stained with antibodies against γ-tubulin (Sigma) and Eg5 staining was carried out with either Eg5 anti-motor domain or anti-tail domain and an anti-rabbit Cy3-conjugated secondary antibody. Cells were washed and counterstained with 4,6-diamidino-2-phenoylindole (DAPI, Sigma). Slides were prepared with Slow-Fade antifade mounting medium (Molecular Probes) and sealed with clear acrylic nail polish. Cells were analyzed using a DeltaVision deconvolution microscope system (Applied Precision).

Cell cycle analysis. Cell cycle distributions were analyzed using flow cytometry analysis with standard protocols. Cells were gently trypsinized, collected, and rinsed in PBS and resuspended in 0.3 mL 50% FBS (Invitrogen) in PBS. While gently mixing, 0.9 mL ice-cold 70% ethanol was added and cells were fixed at 4°C overnight. Cells (1 × 10^6) were washed twice with PBS and resuspended in 1 mL propidium iodide staining solution (200 mg/mL propidium iodide, 0.1% w/v Triton X-100, 2 mg DNase-free RNAse A) and incubated at 37°C for 15 min. Cells were analyzed by flow cytometry using a BD Cultiol EPICS XL flow cytometer (BD Biosciences) at an excitation range of 488 nm, and a 620 band-pass filter for propidium iodide. The percentage of cells in G1, S, and G2-M was based on 20,000 cells counted in triplicate for each line analyzed, and aggregated cells were gated out.

Karyotyping and analysis of ploidy. Cytogenetic analyses were carried out on MEFs using standard methodology. In brief, 1.5 × 10^6 early passage MEFs (P2–P4) were cultured overnight in DMEM supplemented with 10% FBS. Eighty percent confluent cells were cultured in 0.40 g/mL colcemid (Invitrogen) for ~14 h. After colcemid treatment, cells were harvested using a standard protocol, in brief, treated with 0.075 mol/L hypotonic potassium chloride solution and fixed with Carnoy’s fixative (3:1 methanol/acidic acetic). Chromosome spreads were prepared on glass slides, digested with trypsin, and stained with Giemsa for G-banding. Karyotyping was assessed by an experienced cytogeneticist blinded to the sample genotypes.

Proteins of cell lysates. In brief, after two washes with ice-cold PBS, cells were lysed in ice-cold cell lysis buffer [10 mM/L Tris-HCl (pH 7.4), 1% Triton-X, 150 mM/L NaCl, 5 mM/L EDTA (pH 8.0), 1 mM protease inhibitor cocktail (Roche)]. After 30 min of incubation on ice, lysates were centrifuged at 1,000 × g for 10 min at 4°C. The protein concentration of the lysates was determined using the Bio-Rad detergent-compatible protein assay.
Results

Generation of Tg(Pim1-Eg5)Jus transgenic mice. Mice overexpressing mouse Eg5 were generated in the FVB/NJ background using the pPim1Eµ-Eg5 transgene. In this transgene, Eg5 is expressed from the mouse Pim1 promoter and the lymphoid-specific Eµ enhancer (Fig. 1A). It has previously been shown that the Pim1 promoter is capable of driving transcription of cDNAs in the lymphoid tissues. Additionally, the inclusion of the MuLV long terminal repeat (LTR) was incorporated to further boost expression of the transgene (29, 30). A total of nine transgenic lines (Eg5-1 to Eg5-9) were identified by Southern blotting and PCR genotyping. Two lines failed to pass the transgene to offspring and two additional lines displayed low inheritance of the transgene, potentially due to defects in sperm motility and development (data not shown). Five transgenic lines displayed proper transgene inheritance and three were chosen for further study. Seventy mice from two of the transgenic lines (Eg5-2 and Eg5-3), 30 mice from a third line (Eg5-1), and 50 wild-type mice collectively were aged and observed for the development of neoplasias.

Expression analysis. As shown in Fig. 1B, RT-PCR revealed that transcripts from the Eg5 transgene were expressed in multiple tissues of mice from the three lines. Detection of transgenic Eg5 transcripts was carried out using primers to the 3' end of endogenous Eg5 and the MuLV LTR contained in the transgenic construct. Expression in nonlymphoid tissues reflects, in part, constitutive expression driven by the Pim1 promoter and also some contribution of lymphoid infiltrate into other organs.

QRT-PCR analysis was carried out on multiple tissues from both wild-type and transgenic mouse lines to measure overexpression of

![Diagram](image-url)

Figure 1. Tg(Pim1-Eg5)Jus transgenic mice and transgene expression. A, transgenic construct used for pronuclear injection of fertilized eggs. KpnI restriction enzyme sites were used for Southern blotting, and the 3' Eg5-LTR probe (916 bp) was generated by PCR amplification using genotyping primers (horizontal line). B, expression of transgenic Eg5-MuLV LTR fusion transcript was detected by RT-PCR in multiple tissues of three transgenic lines (Eg5-1, Eg5-2, and Eg5-3). Expression in nonlymphoid tissues reflects, in part, constitutive expression driven by the Pim1 promoter and also some contribution of lymphoid infiltrate. WT, wild-type. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. QRT-PCR analysis of transgene expression in transgenic organs (C) and MEFs (D). Breast and lung tumors collected from transgenic animals also displayed overexpression of Eg5. Total RNA was analyzed for levels of total Eg5 expression and fold expression for transgenic organs and tumors was calculated relative to Eg5 expression levels in respective wild-type tissues. Wild-type expression was arbitrarily set at 1; values >1 represent overexpression, whereas values <1 represent loss of expression. C, values were normalized against 18S RNA expression to control for sample load variations.
Eg5 in Tg(Pim1-Eg5)hus mice. QRT-PCR expression analysis detects both endogenous and wild-type levels of Eg5 expression. Therefore, levels of total Eg5 expression shown in Fig. 1C represent the amount of overexpression in transgenic tissues relative to wild-type basal levels in the respective tissues, where expression in wild-type tissues is designated as 1. Eg5 overexpression was observed in transgenic lines Eg5-1, Eg5-2, and Eg5-3 with most tissues displaying ≈4-fold overexpression when compared with wild-type littermates (Fig. 1C). Lung and liver tissues displayed 60-fold overexpression in multiple transgenic lines, with brain and kidney also showing ~15-fold overexpression compared with wild-type. Eg5 levels in transgenic MEFs were observed to be four and eight times the normal level of expression in lines Eg5-2 and Eg5-3, respectively, when compared with wild-type MEFs (Fig. 1D).

To determine if overexpression of the Eg5 transgene produced functional protein, Tg(Pim1-Eg5)hus mice were crossed to Eg5 knockout mice, in which loss of endogenous Eg5 activity results in early embryonic lethality (31). The embryonic lethality phenotype was rescued in Eg5−/− mice carrying the pPim1Eg5 transgene (Supplementary Table S1). Although the transgene rescued the embryonic lethality, the rescue was partial because most rescued mice died within 3 months of birth. These data suggest that biologically functional Eg5 protein was produced from the transgene, although it was likely not expressed in all tissues at normal levels. Eg5 protein was also detected by Western blot analysis of cell extracts prepared from transgenic tissues and MEFs (Supplementary Fig. S4).

**Pim1-Eg5 transgenic mice have increased tumor incidence.** Pim1-Eg5 mice were aged and observed for the development of tumors. The three transgenic lines, Eg5-1, Eg5-2 and Eg5-3, displayed a higher incidence of tumor formation than wild-type with some mice developing neoplasms as early as age 3 months (Fig. 2A). Aged mice developed a broad spectrum of tumor types, with a mean age of onset of 21.1 months. Transgenic line Eg5-3 displayed the highest incidence of tumor formation at 31.4% with incidences in lines Eg5-1 and Eg5-2 of 26.7% and 15.7%, respectively (Fig. 2B). Histologic studies revealed multiple hematopoietic neoplasias, including megakaryocytic leukemias with proliferation of immature megakaryocytes (Fig. 2C), and both marginal zone and follicular B-cell lymphomas. One transgenic mouse with a spleen weight of 1.08 g (wild-type spleen, 0.09 g) was found to have a hemangiomia with both large and small vessels and a proliferative population of endothelial cells lining the smaller vessels. Hyperplasia of the spleen was also observed in multiple mice from lines Eg5-1, Eg5-2, and Eg5-3. Spleens of some mice exhibited myeloid hyperplasias consisting of myeloid expansion with little or no erythroid presence. Others were found to have red pulp hyperplasia with increased numbers of megakaryocytes and erythroid cells combined with expanded B-cell marginal zones. Early lymphoid infiltration of the lung and liver was also observed in many mice with primary splenic neoplasms. Nonhematopoietic lesions were also observed in moribund mice. Pulmonary adenocarcinomas and alveolar adenomas were identified in mice that presented labored respirations. Multiple mice from lines Eg5-1, Eg5-2, and Eg5-3 developed bulging palpable masses near the fore and hind limbs in a bilateral manner, which upon histologic analysis were diagnosed as mammary adenocarcinomas containing highly malignant populations of cells (Fig. 2D). Additional neoplasms were collected and identified in transgenic mice, including neurofibroma/schwannomas, teratomas, and ovarian/myometrial proliferations and neoplasias. Some mice developed independent tumors in more than one tissue type. Additional phenotypes consisting of neurologic abnormalities, megacystis, and dermatitis were also observed and resulted in further reduced survivability, which may correlate with high levels of Eg5 overexpression in organs such as brain (Supplementary Fig. S5).

QRT-PCR expression analysis of tumors collected from transgenic lines revealed substantially higher levels of Eg5 expression relative to their respective wild-type tissues. Mammary adenocarcinomas displayed 90-fold overexpression and pulmonary adenocarcinomas showed 35-fold overexpression (Fig. 1D). Although transgenic liver, kidney, and brain tissues also displayed significantly higher levels of Eg5 overexpression, these organs did not display a significantly higher incidence of tumor formation. Liver pathology showed an increase in megakaryocytic and lymphoid infiltration.

**Eg5 overexpression leads to abnormal spindle formation.** To explore potential effects of Eg5 overexpression on bipolar spindle formation, we assessed the status of mitotic spindles in early passage MEFs (P2–P4) from the transgenics. Immunolocalization of β-tubulin to identify microtubules and staining of DNA was used to study >500 mitotic events. We observed the presence of monopolar spindles in ~8% of mitoses of wild-type cells, whereas they were found in 32.9% of mitoses of cells from line Eg5-3 (P < 0.00001) and 29.8% of mitoses of cells from line Eg5-2 (P < 0.00001; Fig. 3A and B). Cells with monopolar spindles exhibited monoastral arrays of microtubules with condensed chromatin near the periphery of the array. Cells with bipolar spindles had two centrosomes separated by a lattice of interpolar microtubules and condensed chromosomes aligned at the metaphase plate. Immunostaining for Eg5, which also localizes to the mitotic spindle, confirmed the high rate of monopolar spindles in transgenic MEFs detected by β-tubulin staining (data not shown). Our observations of monopolar spindles in cells overexpressing Eg5 recapitulated spindle defects seen previously in cells treated with the small-molecule inhibitor of Eg5, monastrol, and in cells with down-regulation of Eg5 induced by RNA interference (27, 28).

In addition to monopolar spindles, cells from Eg5 transgenic mice had a higher frequency of multipolar spindles. The percentage of multipolar spindles was significantly higher in cells from line Eg5-3 (5.74% ± 0.15%; P < 0.0001) and line Eg5-2 (3.68% ± 0.13%; P < 0.0001) than cells from wild-type mice (0.93% ± 0.4%; Fig. 3C and D). The structure of multipolar spindles varied, ranging from three to five spindle poles in various spatial arrangements with captured chromosomes at the spindle equator. Increased DAPI staining of chromatin associated with multipolar spindles suggests that multipolar spindles may also be associated with polyploidy.

**Overexpression of Eg5 leads to defects in chromosomal segregation.** Recent observations have shown that defects in mitotic spindle development can lead to chromosome missegregation (32, 33). The spindle checkpoint normally serves to prevent mitosis from progressing beyond metaphase when chromosomes fail to properly attach to a bipolar spindle, as would occur when spindle abnormalities such as monopolar spindles are present (10). To determine whether these mechanisms are intact in unselected Eg5-overexpressing MEFs, we assessed gross ploidy status in these cells by flow cytometry. Flow cytometric analyses revealed that perturbation of normal spindle development due to Eg5 overexpression in these unselected cells resulted in accumulation of
cells with a 4N DNA content. The frequencies of MEFs with 4N DNA from lines Eg5-2 and Eg5-3 were substantially elevated, 29.2% and 36%, respectively (P < 0.0001), compared with 25.6% of cells from wild-type mice. The percentage of cells containing 8N DNA content was also elevated in transgenic MEFs, with 4.7% of cells from line Eg5-2 (P < 0.01) and 10.4% of cells from line Eg5-3 (P < 0.0005) displaying octaploidy compared with 2.6% of cells from wild-type mice. These observations further suggest that disruption of mitotic spindle development leads to defects in chromosomal segregation.

Conventional G-banding of metaphase chromosomes from early passage metaphase–selected MEFs (P2–P4) also revealed an increase in the number of cells with elevated DNA content in transgenic cells compared with wild-type cells. In this experiment, cells are treated with colcemid, which causes microtubule depolymerization to arrest the cells at metaphase. Diploid cells (39–41 chromosomes) accounted for 63.4% of wild-type metaphase spreads, compared with 50% of spreads from line Eg5-3 and 30% of spreads from line Eg5-2. Octaploid populations (79–81 chromosomes), however, were significantly higher in transgenic lines Eg5-3 (50%, P < 0.05) and Eg5-2 (66.7%, P < 0.0001) than in wild-type cells (33.3%; Fig. 5B). These observations further suggest that disruption of mitotic spindle development leads to defects in chromosomal segregation.

Multiple models exist to suggest that tetraploidization may be a precursor intermediate of aneuploidy during tumor development (34). Analysis of DNA content in tumor cells overexpressing Eg5 may show whether spindle defects and a resulting...
tetraploidization can lead to the development of aneuploidy. Primary cell cultures generated from transgenic tumors developed distinct foci when cultured under normal conditions. Flow cytometry analysis for DNA content revealed widespread chromosomal missegregation in these tumor cell lines (Fig. 4C and D), as large populations of cells containing 2N to 4N and greater than 4N DNA content were observed. Genomic instability is thought to be a major driving force in the multistep evolution of carcinogenesis and these observations are characteristic of general aneuploidy and chromosomal instability observed with most solid tumors.

Discussion

Cells have evolved highly complex surveillance mechanisms to maintain genomic integrity during mitosis. Cell cycle checkpoints have been established to monitor progression through mitosis, and mitotic arrest at these checkpoints, due to defects, allows for either correction of the defect or entrance into the apoptotic pathway, ultimately resulting in cell death (10). Circumvention of these checkpoints in the presence of mitotic defects results in premature exit from mitosis, which often leads to chromosomal missegregation, aneuploidy, and transformation (35). In this article, we have described a mouse model in which overexpression of Eg5 results in perturbation of mitotic spindle assembly, chromosome instability, and ultimately tumorigenesis. Although Eg5 overexpression was initially identified in hematopoietic malignancies from AKXD RI mice, transgenic mice overexpressing Eg5 displayed a mixed array of tumor development, predictably due to widespread transgene expression and lymphoid contribution to affected tissues. Primary cell lines overexpressing Eg5 displayed monopolar and multipolar spindles as well as changes in DNA ploidy, with the latter two

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Effects of Eg5 overexpression on spindle development in MEFs. Early-passage MEFs (P2–P4) were collected by mitotic shake off, fixed, and stained with β-tubulin (to visualize microtubules) and DAPI (to visualize DNA). A and B, transgenic MEFs displayed significant accumulation of monopolar spindles with microtubules radiating outward from duplicated centrosomes (monoasters) and chromosomes located around periphery of monoasters. C and D, increased incidence of multipolar spindles was observed in transgenic MEFs compared with wild-type. Mitotic spindles with three to five spindle poles were observed by immunocytochemistry. Observations were based on 2,500 mitotic events for wild-type MEFs and 3,500 mitotic events for transgenic (TG) MEFs (P < 0.001 for monopolar spindles and P < 0.001 for multipolar spindles based on Student’s t test).
observations representing clear signals of abnormal progression through the cell cycle.

Cells that have activated spindle checkpoints are prevented from entering anaphase until all replicated chromatids have properly attached to a functional bipolar spindle. Most mammalian cells, however, display a phenomenon termed mitotic slippage, in which prolonged arrest due to the inability to correct problems and satisfy the spindle checkpoint results in “adaptation” or “slippage” of checkpoint arrest and leads to cells with tetraploid genomes (36). Recent work has shown that spindle checkpoint defects often lead to genomic instability similar to that seen in many solid cancers (10). Although genomic instability may arise from mutations in any of the numerous checkpoint components (37), mutations in spindle checkpoint genes are rare in human cancers.

Aneuploidy and chromosome instability are hallmarks of a majority of human cancers (7). For many years, these forms of genetic instability have yielded numerous models suggesting their involvement in cancer (6, 11, 38). Although the gene mutation hypothesis suggests that cancer is caused by mutations, several cases have been presented in solid cancers to refute the explanation of gene mutation as a conclusive cause of cancer (39, 40). An alternative mechanism, termed the aneuploid hypothesis, predicts a two-stage mechanism for tumor initiation. The first stage consists of an event that promotes chromosomal missegregation and aneuploidy; whereas in the second stage aneuploidy destabilizes the genome and promotes an autocatalytic karyotype evolution, resulting in preneoplastic and eventually neoplastic environments (5, 7). The imbalance that aneuploidy imparts on components of the mitotic spindle would result in genomic instability that would explain the heterogeneous karyotypes observed in most solid cancers. Although both models are scientifically valid, in our case of Eg5 overexpression, the aneuploid hypothesis would allow for an initial clonal mutation (i.e., spindle defects), which would initiate additional mitotic defects resulting in aneuploidy. The global effects of aneuploidy would then give rise to polyclonal mutations leading to tumor initiation. Aneuploidy therefore serves as a global stimulus to tumorigenesis either by increasing the chances of loss of heterozygosity of a tumor-suppressor gene, or by amplifying an oncogene through chromosomal duplication. Given the small number of single gene mutations currently proven to instigate the tumorigenic pathway,

Figure 4. Overexpression of Eg5 results in chromosome missegregation. Analysis of ploidy in early passage (P2–P4) unselected MEFs by propidium iodide staining and flow cytometry analysis. A and B, accumulation of tetraploid cells was observed in both transgenic lines compared with wild-type MEFs (n = 120,000 events per line; P < 0.0001, analyses were carried out in triplicate on two embryos from each genotyped line). C and D, analysis of tumor cell lines generated from transgenic tumors revealed widespread aneuploidy and genomic instability. Both transgenic tumor cell lines displayed significant increases in sub-4N and >4N populations of cells.
our observations of increased tumor incidence associated with Eg5 overexpression are of significant interest. Furthermore, based on the broad tissue origin of our observed neoplasms, a global mechanism of tumorigenesis with aneuploidy as an early initiating step would provide a more plausible role for Eg5 overexpression in tumorigenesis.

**Eg5 overexpression imparts an imbalance of forces generated on microtubules of the mitotic spindle.** Key events in mitosis include the formation of a bipolar spindle and accurate segregation of chromosomes to daughter cells. These events are dependent on microtubule rearrangement mediated by microtubule motors that bind to and move unidirectionally along microtubules (1, 2, 41). In early *Drosophila* embryos, spindle pole separation and elongation occurs throughout mitosis in a nonlinear fashion, as net forces generated upon microtubules of the poles fluctuate through various phases of the cell cycle. Cytoplasmic dynein, a minus end–directed microtubule motor, imparts motor forces on astral microtubules of the centrosomes, promoting their migration along the outer edge of the nuclear membrane (Fig. 6A). These forces, however, seem to be opposed by microtubule forces generated by additional molecular motors, such as Ncd, which may serve as a brake during the early phases of spindle pole migration. As early spindle pole migration progresses, it is suggested that KLP61F, an Eg5 homologue, and dynein cooperate to further drive spindle pole separation, whereas Ncd continues to antagonize these motors by pulling centrosomes together (Fig. 6B). Studies in early *Drosophila* embryos on microtubule motors using inhibitors of dynein, Ncd, and KLP61F show that spindle pole migration and elongation are controlled by the unique balance of forces generated by these three motors (42–44). Similarly, antagonistic forces have been shown where human microtubule motor homologues, cytoplasmic dynein, HSET, Eg5, also disrupt microtubule organization and spindle dynamics (45).

Our data show that overexpression of Eg5 disrupts normal spindle development in cells of transgenic mice. With current evidence supporting the need for microtubule force balance (46), it is likely that overexpression of Eg5 results in an imbalance of these forces, resulting in the development of abnormal spindles. One proposed mechanism involves excess Eg5 motor activity on the interpolar microtubules of separated centrosomes. Excess Eg5 activity would lead to a force imbalance and an increase in outward force resulting in premature spindle elongation and overshooting of centrosome separation (Fig. 6C). Net forces imparted on mitotic spindles with excess Eg5 activity would theoretically resemble a net force imbalance similar to those observed in metaphase spindles with loss of minus end–directed motors, such as Ncd or dynein. Knockdown or complete loss of either of these two motors results in spindle defects and chromosomal missegregation (47, 48) resembling abnormalities seen in our transgenic mice.

Spindle defects normally trigger activation of the spindle checkpoint due to failure of duplicated chromatids to attach to a functional bipolar spindle and subsequently prevent entry into anaphase until the abnormalities are corrected or the cell enters the apoptotic pathway to prevent chromosomal missegregation. “Slippage” through the spindle checkpoint and progression through anaphase would result in tetraploid G1 cells, and reentry into mitosis could result in a second round of DNA replication generating an octaploid DNA content in addition to centrosomal duplication, resulting in the development of multipolar spindles followed by possible chromosomal missegregation and aneuploidy (12). With the number of potential mitotic defects likely to occur in the life span of an average cell, it is likely that spindle checkpoints will become activated and cell cycle arrest will ultimately result in either resolution of the abnormality or apoptotic death. Any stimulus to the rate and incidence of mitotic defects would increase the opportunity for mitotic slippage and the survival of an initiating mutation that may lead to further genomic instability and ultimately tumorigenesis. The long latency of Eg5 tumors indicates that although Eg5 overexpression perturbs spindle dynamics, activation of the spindle checkpoint does occur. Over time, however, some cells are able to circumvent these surveillance mechanisms.

**Figure 5.** Karyotype analysis of transgenic MEFs. A, G-banding of chromosomes from wild-type and transgenic metaphase-selected cells revealed a significant increase in the number of polyploid cells. B, graphical representation of the percentage of cells with octaploid genomes.
mechanisms, causing cells to accumulate additional mutations and undergo neoplastic transformation due to genomic instability. Given the complexity of cancer-specific phenotypes, and the failure of Eg5 overexpression to cause neoplastic transformation or induce micronuclei formation in cultured cells (Supplementary Data), a mechanism involving aneuploidy and the alteration of large complements of regulatory and structural genes remains a logical mechanism for the initiation of tumorigenesis. With recent data detailing Eg5 overexpression in many human solid tumors and leukemias (49), in addition to recent work demonstrating a correlation between Eg5 expression and the response of non–small cell lung cancer to antimitic agents used in chemotherapeutic treatments (50), understanding the role of Eg5 in cell division may aid in the development of Eg5 as a potential therapeutic cancer target. Further studies of Eg5 function may benefit from high-resolution, real-time analysis of microtubule motors and spindle dynamics in mammalian cells as done with Drosophila embryos and Xenopus mitotic extracts to understand the molecular mechanism for Eg5 overexpression and its effect on the balance of net forces exerted on microtubules of the mitotic spindle.

Figure 6. A model for spindle development with Eg5 overexpression. The activities of microtubule motors upon the mitotic spindle are carefully orchestrated to maintain dynamic balances of antagonistic forces to organize a sequence of events throughout the progression of mitosis. Key events of mitosis include spindle assembly, maintenance and elongation, and chromosome capture, congression, and segregation. Early in mitosis (prophase and prometaphase; A), bipolar assembly begins before nuclear envelope breakdown with the migration of centrosomes to opposing poles. Minus end-directed motors are proposed to be involved in centrosome migration. One potential mechanism for Eg5 overexpression leading to spindle defects may occur later in mitosis after centrosome migration. B, Eg5 normally functions in microtubule overlap (interpolar microtubules) at the metaphase plate to stabilize the spindle before anaphase to allow for chromosome capture and attachment to spindle. A force balance would occur at this stage where outward forces (green arrows) and inward forces (red arrows) result in an equal net balance of opposing forces preventing spindle elongation. Following chromosome capture, Eg5 motor activities generate additional outward forces (green arrows) upon the microtubules to allow spindle elongation and chromosome segregation to opposing poles. C, in our model of Eg5 overexpression, excess Eg5 would generate additional outward forces (green arrows) throughout mitosis but more importantly before anaphase, causing premature spindle elongation and overshooting (yellow arrows), thereby preventing the attachment of chromosomes to the spindle microtubules. Excessive spindle separation may then lead to collapse of spindles and the formation of monopolar spindles.
References


Overexpression of *Eg5* Causes Genomic Instability and Tumor Formation in Mice

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