THE CONSEQUENCES OF MITOTIC SEGREGATION DEFECTS IN ORAL CANCER CELLS AND THEIR CONTRIBUTION TO CHROMOSOMAL INSTABILITY

by

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ABSTRACT

Mitotic segregation defects such as multipolar spindles, anaphase bridges, and micronuclei have long been observed in cancer cells, but it is not known whether these defects lead to aneuploidy or even contribute to tumorigenesis. We visualize living oral squamous carcinoma cells with stable expression of GFP-histone H2B fusion. Expression of this fusion protein labels chromosomes clearly and does not disrupt the cell cycle, alter the doubling time or produce any defects previously unseen in fixed cells. These carcinoma cells survive the formation of anaphase bridges and micronuclei and complete a second cell division in the same amount of time as unaffected cells. Micronuclei were formed after every division that contained an anaphase bridge in cells we examined. Most often, each daughter cell contained a micronucleus. These results suggest that the chromosome breaks at multiple points along its length and breaking may not be due to a "tug of war" between spindle poles. The movement of micronuclei was very dynamic compared to the nuclei during interphase and micronuclei do not appear to be transcriptionally active. Using long-term live cell imaging we were also able to observe the fate of these cells through two divisions and have determined the length of each phase of mitosis. Anaphase bridges and lagging metaphase chromosomes both lengthen mitosis,

suggesting that the mitotic spindle checkpoints are at least partially active in cells. The mitotic delays occurred during metaphase in these defective cells. We have also analyzed centrosomal components including the mitotic apparatus protein, NUMA. No correlations were found between protein expression of NuMA and gene amplification or segregation defects. In summary, we have shown that cells continue to proliferate after the occurrence of mitotic defects and these defects contribute to chromosomal instability.

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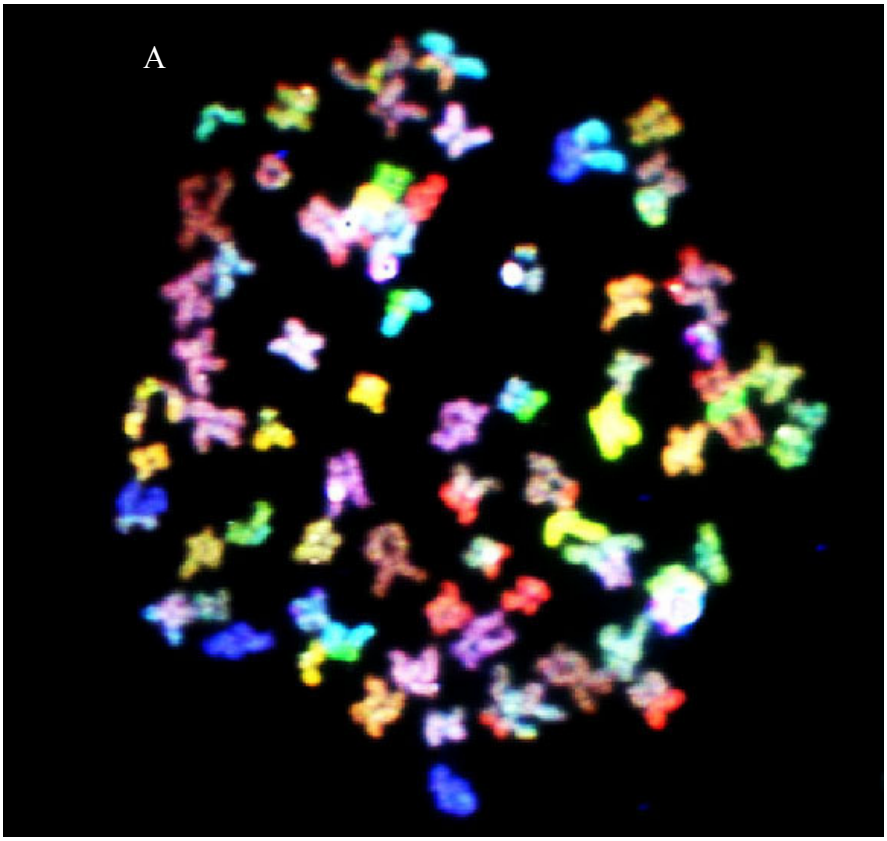
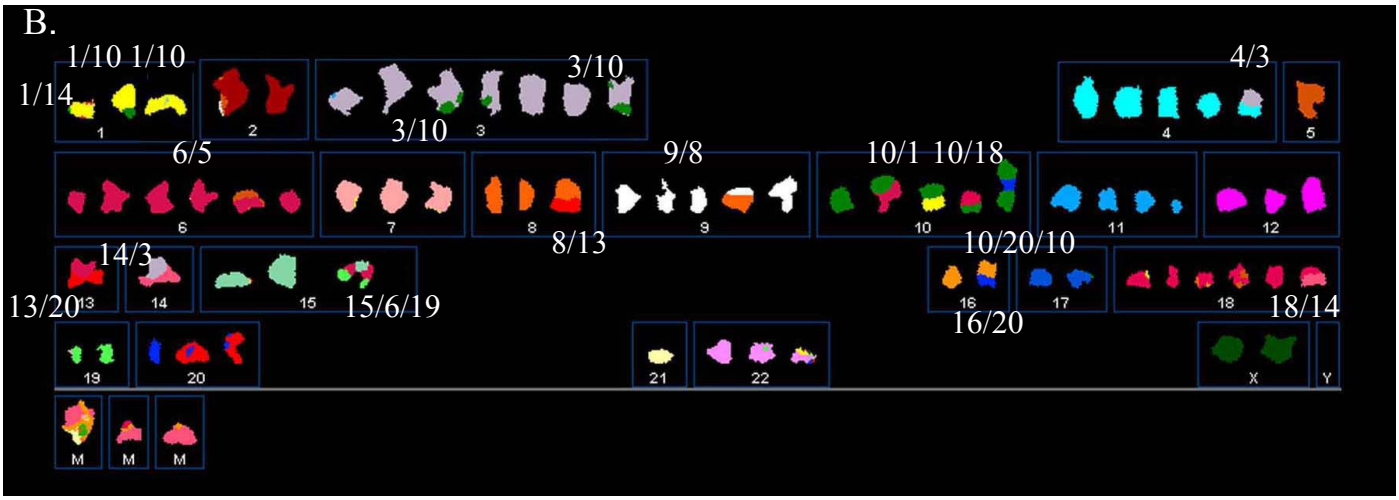
CHAPTER I. INTRODUCTION

The goal of cell division is to produce two daughter cells with an identical genetic composition. Cancer cells are unable to do this reliably. Virtually all solid tumors are aneuploid, that is, they have changes in chromosome numbers and structure (Duesberg and Rasnick, 2000). A representative spectral karyotype (SKY) of an oral cancer cell is shown in figure 1. This image reflects what is commonly seen in tumor cells; an abnormal chromosome number with a gain or loss of one or more chromosomes from the normal diploid karyotype. Cell karyotypes vary from cell to cell in both tumor tissue and tumor cells grown in culture (Saunders et al. 2000). This variability is known as chromosomal or genomic instability. Over one hundred years ago it was proposed by Theodor Boveri that mitotic segregation defects were the cause of the aneuploidy seen in cancer cells and that these defects are responsible for producing irreparable and tumor-like conditions in sea urchin embryos (reviewed by Surridge 2001). But the mechanism for producing this chromosomal instability and aneuploidy is still unknown and conflicting hypotheses still exist (reviewed by Jallepalli 2001). It has been proposed that aneuploidy begins without previous mutation and is self propagating (Duesberg and Rasnick 2000). In this model, carcinogens damage the spindle or fragment chromosomes to produce aneuploidy, which then leads to new and evolving karyotypes and further instability with each subsequent cell division. A more commonly accepted model is that individual genetic mutations in key genes at the level of base pair changes are the initial events, and an accumulation of these mutations eventually leads to tumorigenesis and genetic instability (Lengauer, Kinzler et al. 1997). This relatively well characterized model proposes that mutations must occur in genes that lead to errors in key pathways involved in proliferation and repair, and although some genetic changes are very common, no specific or consistent mutations have been found in all tumors (Duesberg and Rasnick 2000). I favor a model combining these two proposals. Damage may induce segregation defects, which then lead to aneuploidy and subsequent genetic mutations, deletions or amplifications. As the defects may contribute to chromosomal instability, cells with particular genetic mutations may have a survival advantage and eventually produce neoplastic growths. I am interested in determining whether the segregation defects seen in tumor cells also play a role in chromosomal instability.

Figure 1: Spectral karyotype of UPCI:SCC40 cell.

Aneuploidy is evident in this typical oral cancer cell. **A**: Spectral karyotyping (SKY) uses chromosome specific dyes to distinguish between each chromosome by spectral imaging. Whole chromosomes and segments are either amplified or lost. Easily identifiable translocations are labeled in **B**. SKY performed by Shalini Skarja and Susanne Gollin.





Normal cell division requires the following for proper segregation: a properly formed spindle apparatus, mechanisms to move chromosomes including the attachment of microtubules to kinetochores, chromatid condensation, sister chromatid cohesion, a mechanism for detecting a bipolar attachment, and a mechanism for separating sister chromatids once they all have bipolar attachments (Nasmyth 2001). Each process must be properly executed or unbalanced segregation will be the result. The components required for normal cell division are described in figure 2.

The cell cycle is a sequential and unidirectional process. Diffusible chemical signals are the controlling mechanisms for the regulation of the onset of each phase (Rao and Johnson 1970). These signals also prevent improper entry into or exit from each different phase. Subsequent work has shown that two critical events in the cell cycle are the most important in cell division; the initiation of S phase and the initiation of mitosis. The control of these events is accomplished by the regulation and activation of cyclin-dependent kinases. The regulation of this process is crucial in preventing errors in segregation.

Cell cycle checkpoints also ensure accurate segregation during division. Checkpoints are regulatory feedback mechanisms that assure that entry into the next step of the cell cycle does not occur if the previous step is incomplete or unsuccessful (Hartwell and Weinert 1989). The DNA damage checkpoints cause cell cycle arrests during G1 or G2. These checkpoints give the cells time to repair spontaneous DNA damage or damage caused by exposure to ultraviolet rays or harmful chemicals (reviewed by Greenwood 2001). The first identified checkpoint protein, RAD9, mediates a cell cycle arrest following irradiation and allows the repair of DNA damage prior to division (Weinert and Hartwell 1988). Many checkpoint proteins have been discovered since, but how the damage is recognized and how the signal to arrest is carried throughout the cell is not fully known. The checkpoint pathways are very complex causing more then just an arrest in the cycle, but also the activation of many other cellular activities including: DNA repair pathways, chromatin remodeling, movement of repair proteins to the sites of damage, and activation of transcription (reviewed by Zhou and Elledge 2000).

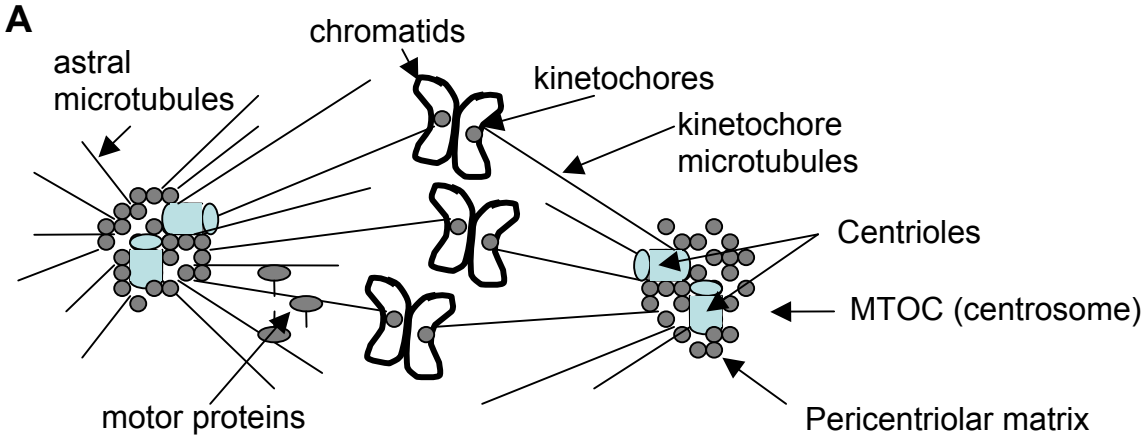
Figure 2: The mitotic apparatus.

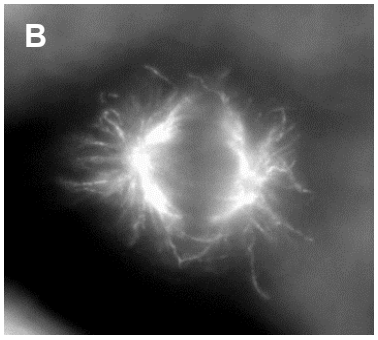
A: An illustration of a metaphase cell. The microtubule organizing center (MTOC) contains centrioles and pericentriolar material composed of γ-tubulin and pericentrin which nucleate microtubules to form the astral, kinetochore, and polar microtubules. Condensed sister chromatids are shown aligned on the metaphase plate with the kinetochores on each chromatid attached to kinetochore microtubules. Molecular motors are required to maintain the spindle apparatus and to move the segregating chromosomes.

B: the spindle apparatus visualized with β tubulin staining (described in Table 1),

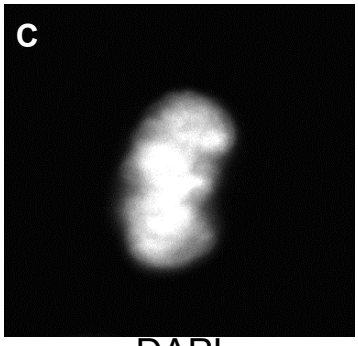
C: DNA-specific fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) staining of the metaphase chromosomes,

D: kinetochores visualized with anti-CREST autoimmune serum (described in Table 1).

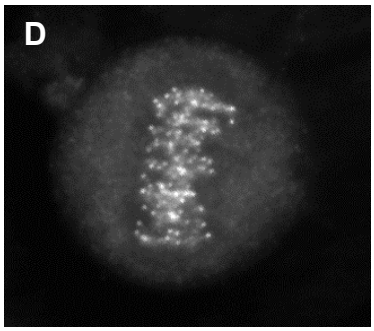




anti-β tubulin



DAPI



anti-kinetochore

Another checkpoint mechanism for ensuring accurate division is the spindle checkpoint (also called the mitotic checkpoint) first characterized on a genetic level in two separate studies using *Saccharomyces cerevisiae*. The spindle checkpoint causes a cell cycle arrest during mitosis and is mediated by the MAD (mitotic arrest–deficient) and BUB proteins (budding uninhibited by benzimidole) (Li and Murray 1991; Hoyt, Totis et al. 1991). These proteins were shown to be essential for mitotic arrest or progression. It is now known that the MAD/BUB checkpoint leads to cell cycle arrest by inhibiting the anaphase promoting complex (APC) which is involved in mediating separation of sister chromatids (Jallepalli 2001). The spindle checkpoint mechanism senses proper kinetochore attachment and tension and prevents the activation of the proteins involved in anaphase onset if defects are present. Daughter cells with uneven numbers of chromosomes will be the result if anaphase is initiated before proper bipolar attachments have been made.

An example of a cancer caused by a defective spindle checkpoint is a rare condition called premature centromere division-related variegated aneuploidy (PCD). This condition leads to very early development of cancer with 5 of 6 patients developing tumors as infants (reviewed by (Plaja, Perez et al. 2001). PCD causes a high percentage of cells to be aneuploid. This condition is caused by a defect in the mitotic spindle checkpoint (Matsuura 2000). Which regulatory mechanisms are disrupted in the majority of other cancers or if such a disruption is what initiates tumorigenesis is not known. Understanding the roles of checkpoints in tumorigenesis may provide insight into new and effective therapies for cancer.

We are investigating mitotic segregation defects and possible checkpoint defects in cancer cells and whether these defects are involved in perpetuating chromosomal instability. We use oral squamous-cell carcinoma (OSCC) cell lines as a cancer model. Oral cancer is relatively well characterized since tumor tissue and adjacent unaffected tissue are easily accessible. We have characterized segregation defects in OSCC cells in an attempt to determine the relationship between segregational errors and chromosomal instability in both normal and cancer cells. Mitotic segregation defects such as anaphase bridges, interphase bridges, micronuclei, and multipolar metaphases, described in figure 3, have been observed in fixed oral cancer cells. But the fate of cells containing these defects and the consequences of dividing with these defects are unknown and cannot be determined by immunofluorescence analysis of fixed cells. To address this problem, we have observed living cells dividing with segregation defects in real time.

I propose that mitotic segregation defects are a major contributor to chromosomal instability in cancer cells and that these defects may also be the initial event in producing aneuploidy. I am attempting to answer how cells deal with these defects by determining what are the consequences of defects on division and what is the fate of cells containing defects.

Figure 3: Mitotic segregation defects in oral cancer cells.

Mitotic segregation defects visualized with indirect immunofluoresence of fixed OSCC cells (antibodies and stains are described in Table 1).

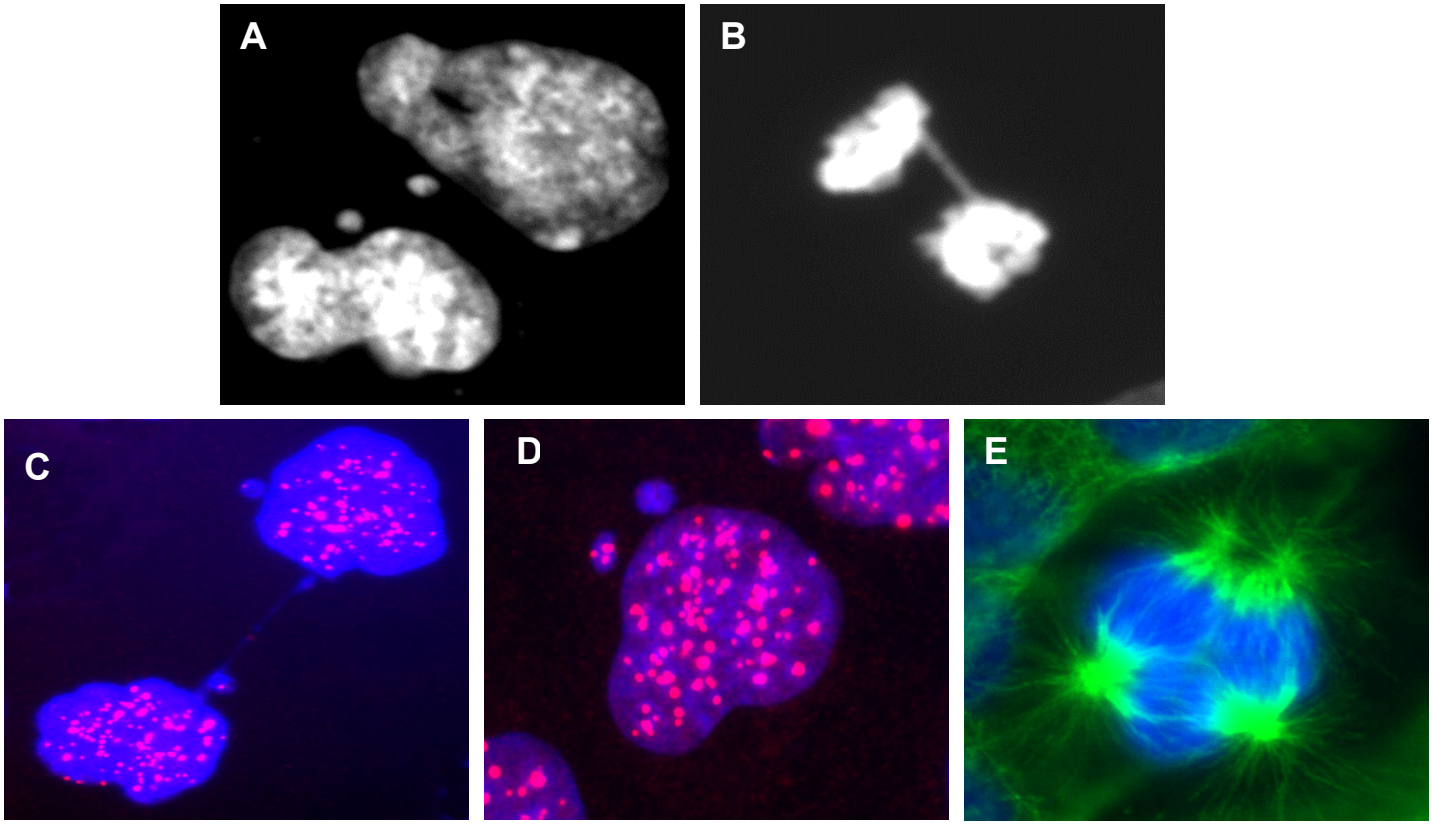
A: micronuclei visualized with DNA specific DAPI stain,

B: an anaphase bridge visualized with DAPI,

C: an interphase chromatin bridge with centromeres visualized by anti-CREST antibody,

D: an interphase cell with two micronuclei, centromeres visualized with anti-CREST antibody,

E: a multipolar metaphase visualized with DAPI and anti-β tubulin antibody.



CHAPTER II. Mitotic segregation defects occur in viable tumor cells in culture.

1. Introduction:

The significance of mitotic segregation defects on tumorigenesis is generally uncharacterized. The mitotic segregation defects we have observed in fixed oral squamous-cell carcinoma (OSCC) cell lines include anaphase bridges, interphase bridges, multipolar spindles and micronuclei. In order to address the question of whether the types of defects seen in fixed cells contribute to the chromosomal instability seen in live cells we first must determine whether defective cells are able to divide and whether their progeny are viable. If cells continue to proliferate after the occurrence of defects we will then attempt to determine the consequences of these defects on individual cells.

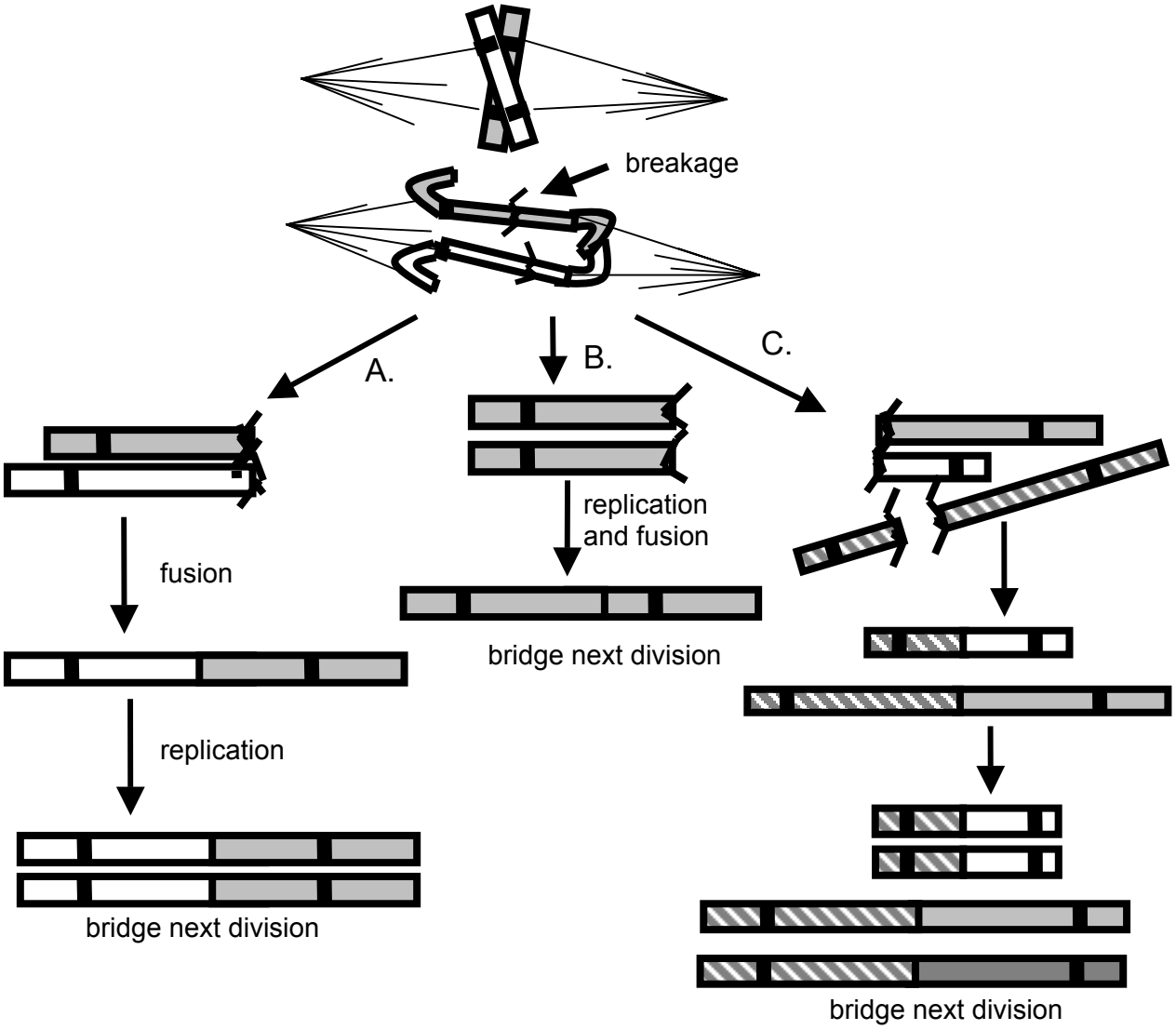
Anaphase bridges occur in many types of cancer cells but it is not known whether these cells arrest or recover and continue to proliferate. We addressed whether bridges break, form micronuclei, get pulled to one daughter cell, or remain in the middle of the spindle to form interphase bridges. Anaphase bridges (shown in figure 3B) can be seen with the DNA specific stain, DAPI, as complete strands of chromatin stretched between the segregating sets of chromosomes and may be evidence of dicentric chromosomes. Dicentric chromosomes may be produced by telomere fusions, DNA repair errors, recombination defects or fusions of chromosome fragments. These dicentric chromosomes may be involved in the breakage-fusion-bridge (BFB) cycle first proposed by Barbara McClintock (1941; figure 4 and discussed in detail in chapter III).

Interphase chromatin bridges may be evidence of unresolved anaphase bridges. These bridges can be seen as complete or nearly complete bridges of chromatin between interphase nuclei (figure 3C). We attempted to determine how these interphase bridges form and their consequences on future divisions.

We would also like to determine whether cells are able to divide and survive after the formation of multipolar spindles (figure 3D). The normal bipolar spindle has been disrupted in these cells by an unknown mechanism. During metaphase, chromatids congress and line up between the extra spindle poles. Multipolar spindles would presumably produce aneuploid daughter cells if they were able to divide.

Figure 4: Breakage-Fusion-Bridge (BFB) Cycle

A model of the BFB cycle illustrating dicentric chromosomes attached to both poles and subsequently breaking during anaphase (adapted from Gisselsson et. al., 2000). These broken chromosomes with telomere-less ends may fuse with each other (**A**) or other telomere-less chromosomes (**C**) as demonstrated by studies in yeast (Moore et al., 2000). Alternatively, the fragments may replicate before fusion and subsequently these replicated telomere-less fragments may fuse to produce a single dicentric chromatid (**B**). These dicentrics can then repeat the cycle; bridging, breaking, and fusing during the next division. Variations of these models have been widely proposed but there is little direct evidence supporting them in mammalian cells.



In order to observe live cells dividing with segregation defects it is necessary to label the chromatin of living cells without disturbing division. The following approaches have been used previously to study chromosomes in living cells: microinjection of fluorescently tagged molecules, incorporation of fluorescent nucleotides into DNA, or green fluorescent protein hybrid proteins (GFP) (reviewed Manders, Kimura et al. 1999). GFP fusions are used as *in vivo* reporters to localize proteins. In order to label many cells at once, to obtain stable labeling throughout successive divisions, and to follow segregation defects in real time, we chose to use a GFP-histone H2B fusion (Kanda, Sullivan et al. 1998). When using GFP-tagged proteins there is the possibility of interference of the large GFP tag with the function of the fused protein, histone H2B in this case. As GFP is fused to the C terminal tail of H2B, it presumably does not interfere with the nucleosome structure. Expression of GFP-histone constructs have been shown to be functional without disrupting cell division (Kanda, Sullivan et al. 1998). A GFP-histone2A fusion was also shown to function in *Drosopholia melanogaster* without disturbing cell function by rescuing a mutant histone lethality (Clarkson and Saint 1999). When visualizing cells with GFP fusions it is also necessary to be aware of possible damage caused by radiation. High doses of radiation, particularly from the UV wavelengths, are toxic to cells (Brakenhoff 1996). Cells may still be motile and be able to replicate DNA but may be too damaged to continue through mitosis. After optimization of our labeling system and data acquisition, we have been able to visualize segregational defects and determine the fate of cells containing these defects. We found that cells containing anaphase bridges, micronuclei and multipolar spindles are able to divide and remain viable until the next cell division. These defects are not simply evidence of arrested and dying cells.

2. Materials and methods:

2.1 Cell lines:

UPCI:SCC 40, 70, 78, 103, 131, 154, and 172 oral squamous-cell carcinoma cell lines were provided by Susanne M. Gollin, Deparment of Human Genetics, University of Pittsburgh. These cell lines are heterogeneous populations of keratinocytes grown from tumor tissue. The patients were not treated with chemotherapy or radiation before surgery. The cells were cultured in M10 medium (minimal essential medium (MEM) supplemented with; 10% fetal bovine serum, 2mM L-Glutamine, 0.05mg/mlGentamicin, and 1% MEM non-essential amino acids (Gibco), grown at 37^0 C with 5% CO_2.

Diploid human fibroblast cells (GM03349B, Coriell Institute, Camden NJ) were cultured at 37^0 C with 5% CO_2 in minimum essential medium (Gibco BRL) supplemented with 15% fetal bovine serum.

Normal human oral keratinocytes (HN-00-172, HN-01-328, 344, and 348) primary cell cultures were provided by Sarita Singh and Susanne M. Gollin, Deparment of Human Genetics, University of Pittsburgh. These cell cultures were established from histopathologically normal tissue from consenting patients undergoing oral surgery who had no evidence of dysplastic growth. Cells were maintained at 37^0 C with 5% CO_2 in keratinocyte growth media (KGM) supplemented with KGM BulletKit (Clonetics, Cambrex Biosciences).

2.2 Indirect immunofluorescence:

Cells were grown on glass coverslips, fixed in methanol for 20 minutes at -20^0 C, then air-dried. Coverslips were then stored at -20^0 C. For immunofluorescence (IMF), fixed cells were rehydrated in PBS with 1%BSA. Primary antibodies (described in Table 1) were diluted in PBS/1%BSA. 75ul of each dilution was added to cells and then incubated in a humid chamber for 1 hour at 37^0 C. Coverslips were washed three times in PBS, incubated with secondary antibodies at 37^0 C, washed again, counterstained with DAPI, and mounted onto slides using antifade mounting solution containing p-phenylene diamine and glycerol. Cells were then viewed with an Olympus BX60 microscope and imaged with a Hamamatsu CCD camera.

Table 1: Antibodies, stains, and reagents:

Reagent	Supplier	Specificity	IMF dilution	Blot dilution
DAPI 4',6-diamidino-2-phenylindole	Sigma	DNA	1ug/ml	
Anti-CREST human auto-immune serum	Gift, C. Feghali, T. Medsger Univ. of Pittsburgh	Kinetochore/ centromere	1:250	
Cy3 goat anti-human IgG	Jackson Immunoresearch	Human 2^0		
Anti-β-tubulin mouse hybridoma	Gift, C. Walsh	β-tubulin	undiluted	1:10
AlexaFluor488 goat anti-mouse IgG	Molecular Probes	Mouse 2^0	1:250	
Anti-γ-tubulin monoclonal antibody	Sigma	γ-tubulin	1:250	1:1000
Anti-NuMA polyclonal antibody	Gift, D. Compton	NuMA	1:500	1:1000
Anti-HSET polyclonal antibody	Gift, D. Compton	HSET	1:250	1:500
Anti-actin polyclonal antibody	Sigma	actin		1:1000
α-amanitin polymerase inhibitor	Sigma	RNA polymerases		
pBOS-H2BGFP vector	Pharmingen	Histone H2B		
AlexaFluor488-5-UTP	Molecular Probes	RNA		

2.3 H2B-GFP expression:

Plasmid preparation: pBOS-H2BGFP vector purchased from Pharmingen constructed by (Kanda, Sullivan et al. 1998). Transformation of competent cells by electroporation, cells grown on LB plates with 0.25ug/ml ampicillin, colonies were selected and grown in LB medium supplemented with ampicillin. Plasmid was prepared with Qiagen plasmid maxi prep kit (#12162) following manufacturers instruction. Plasmid construction was confirmed by restriction digest with XbaI and BamHI restriction enzymes. DNA concentration and purity was determined by spectrophotometry.

Transfections: UPCI:SCC040 cells were transfected using Lipofectamine reagent (Gibco BRL). A mixture of 3ug of DNA, 8ul of Lipofectamine reagent and MEM were added to cells and then incubated for 2 hours at 37^{o}C with 5% CO_2. Cells were rinsed and then incubated for 24 hours in MEM with 10%FBS. To enrich for GFP expressing cells, 5ug/ml of blasticidin S (Calbiochem) was added to the medium for 4 days and then reduced to 2ug/ml for 3 weeks. The optimal blasticidin S concentration was determined by a sensitivity assay in non-transfected cells (Izumi, 1991). Cells were then grown in antibiotic free medium, maintaining ~75% labeling.

2.4 Live cell imaging:

Live cell imaging was performed either at Simon Watkins' Center for Biological Imaging (CBI) University of Pittsburgh Medical Center or in our own lab. UPCI:SCC040 cells expressing H2B-GFP were maintained in a heated Bioptechs perfusion chamber (Cranberry, PA). Time-lapse images were collected on either Zeiss or Nikon microscopes completely automated for live cell imaging with automated xyz stages and dichroics. Data acquisition and analysis was achieved with Metamorph imaging software (Universal Imaging Corp., Downingtown PA).

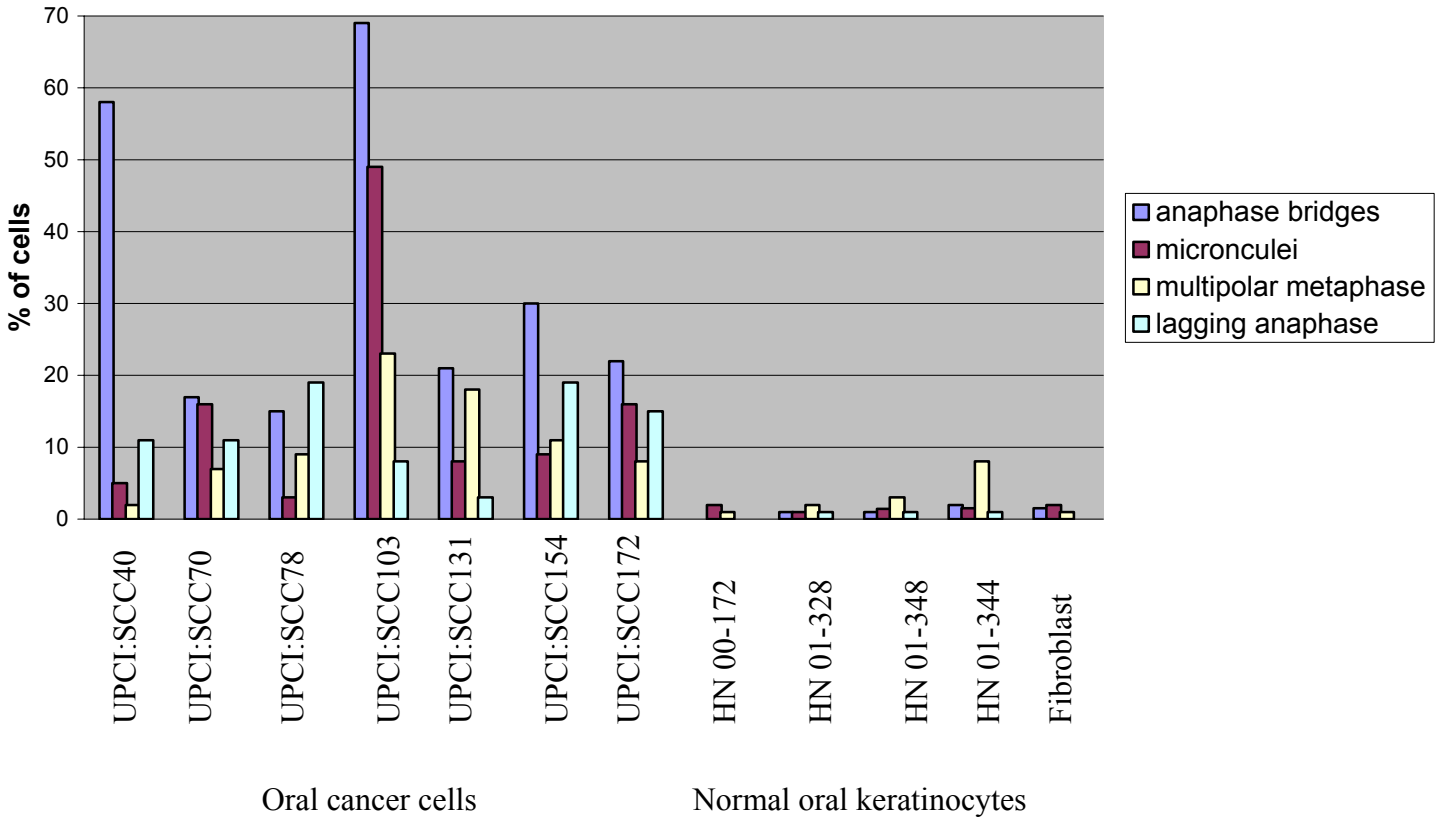
Optimization of environmental conditions: the Bioptechs live cell chamber provided a constant 37^{0}C environment for the cells but did not provide 5% CO_2 humidified air as commonly used in incubators. To provide more consistent pH levels in this closed system, 25 mM HEPES buffer was added to the cell medium. To reduce light exposure and possible cell damage, exposure times were reduced to 100 milli seconds (ms) and a 40X air objective was used for long-term image acquisition. For shorter time sequences, a 100X oil objective was used.

3. Results:

3.1 Segregation defects observed in fixed cells.

In order to determine the types of segregation defects and at what frequencies they occur in normal and cancer cells in culture, we used indirect immunofluorescence of fixed cells. Immunofluorescent images of the different types of defects are shown in figure 3. Metaphases were determined to be multipolar by a combination of DAPI staining to visualize the unusual metaphase plate configurations and β tubulin staining to visualize the microtubule organizing centers. Anaphase bridges were differentiated from lagging chromosomes by reserving the term 'bridge' for only continuous strands of chromatin between the separating chromosomes while other fragments or arms of chromatin were called lagging chromosomes. These lagging chromosomes may have been bridges at an earlier time point but this can not be determined by examination of fixed cells. Interphase bridges are chromatin strands seen by DAPI staining that connect decondensed chromatin between two or more interphase nuclei. Micronuclei are defined as small, distinct, round or oval shaped DAPI stained material that are completely separate from the main nucleus but stain with the same intensity (Fenech 2000). The frequencies of each defect seen in the different types of cell cultures are shown in figure 5.

Figure 5: Frequency of mitotic segregation defects in oral cancer cells, normal oral keratinocytes, and diploid fibroblasts. One thousand interphase cells were examined for micronuclei and one hundred mitotic cells were examined for segregational defects in each cell culture.



Segregation defects were seen relatively often in cancer cells but rarely in cells derived from normal tissue. As many as 87% of cells contained defective anaphase configurations in UPCI:SCC103 oral cancer cells while less than 2% of anaphase fibroblast cells were defective. These results are similar to another study where only 0-2% of anaphase fibroblast cells were observed to contain bridges and as many as 94% of anaphases contained bridges in highly malignant tumor cells (Gisselsson, Jonson et al. 2001). Multipolar metaphases were observed in oral cancer cells at an average frequency of 10%. These defects are not solely artifacts of growing cells in culture as anaphase bridges, micronuclei and multipolar configurations have

been observed in tumor tissue sections (Gisselsson, Jonson et al. 2001; Saunders, Shuster et al. 2000). However, the frequency of defects is difficult to estimate from tissue and may increase during culturing. We examined this possibility by determining the frequencies of defects in cultured cells over time. Defects were seen at consistent rates in each cell line throughout passaging without increasing over generations, implying that culturing does not increase defects. For example, UPCI:SCC172 had similar rates of multipolar metaphases in the eleventh passage, after the culture was established, as it did in the 31st passage (approximately 3-4 days between passages). An average of 8% (+/- 2%) of metaphases were multipolar in every passage examined. Some cell lines had consistently higher frequencies of anaphase bridging than other cell lines suggesting that this defect may be heritable. For example, UPCI:SCC40 had an average of 58% of anaphase cells containing bridges through 20 generations while UPCI:SCC78 had only 15%. Using IMF, we were able to quantify the types of defects but were unable to draw any conclusions about the consequences of these defects on the cells.

Defects were observed at lower frequencies in normal oral keratinocytes. Normal cells were harvested from tissue and grown for 7 to 14 days and then fixed and stained. These primary cultures were derived from tissue obtained from both smokers and non-smokers. The most common defect seen in primary cultures were micronuclei although bridges and multipolar cells were also seen in some cells (figure 5). One primary culture, HN 01-344, contained multipolar cells at the same frequency as some cancer lines. Overall, normal cells contain much lower frequencies of defects, but they do exist.

3.2 Expression of H2B-GFP fusion protein in UPCI:SCC cells.

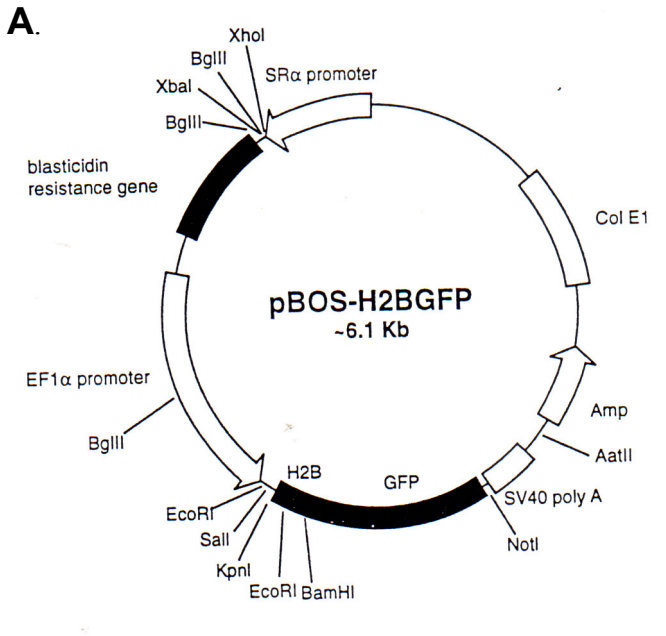
In order to observe living cells with segregation defects, we produced cell lines expressing fluorescently labeled chromatin. Chromatin was labeled by the expression of H2B-GFP (figure 6). Cells were transfected with the H2B-GFP plasmid using Lipofectamine (Gibco). Cultures were enriched for expressing cells by selection with the antibiotic, blasticidin S. When expressed in UPCI:SCC40 oral cancer cells and in fibroblast cells, H2B-GFP localized to interphase nuclei in the same patterns as chromatin stained with DAPI (figure 7, E and F). During mitosis the fusion protein specifically labels condensed chromosomes (figure 7, A-D). When compared to fixed, non-transfected cells stained with DAPI, the H2B-GFP labeled cells

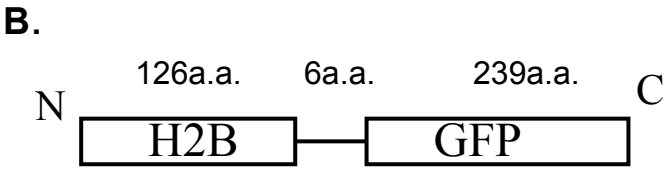
appeared identical during all phases of the cell cycle and no previously unobserved defects were seen. UPCI:SCC40 cells expressing H2B-GFP were compared to their parental non-transfected line (figure 7H) by staining with DAPI and counting 1000 cells for mitotic index and micronuclei. No significant differences in the frequencies of segregational defects were seen between the GFP expressing cells and the parental line. One hundred mitotic cells were examined for defective anaphases (both bridges and lagging chromosomes) and both lines contained approximately 67% defective anaphases. Doubling time was determined and compared by plating equal numbers of cells (6.0×10^5 cells) then trypsinizing and recounting at various time intervals. The doubling time was approximately 26 hours in both cell lines implying the cell cycle is not disrupted by the expression of the fusion protein. The frequency of normal anaphase cells compared to abnormal anaphase cells was also compared in H2B-GFP expressing cells versus H2B-GFP negative cells in the same transfected culture. DAPI staining was used to examine the GFP negative cells. Equal frequencies of anaphase defects and micronuclei were seen suggesting that H2B-GFP expression does not alter the defect frequency. After antibiotic selection, the expression of H2B-GFP was then stable for several months in the absence of selection suggesting that the stable transformants have integrated the gene and this does not markedly change chromosomal stability. Similar results were reported previously (Kanda, Sullivan et al. 1998).

Figure 6: Histone H2B-GFP fusion protein

A: map of the pBOS-H2BGFP vector (Kanda, Sullivan et al. 1998). The plasmid includes the mammalian EF-1α promoter, blasticidin S resistance gene and the H2B-GFP fusion,

B: the H2B-GFP fusion protein





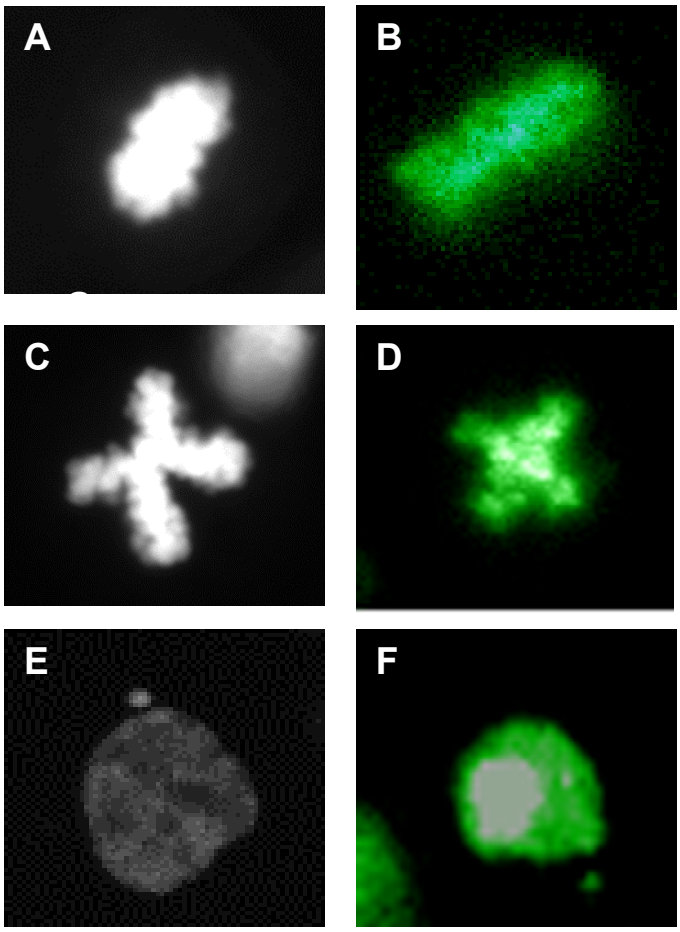


Figure 7: Expression of H2B-GFP fusion protein. Expression of H2B-GFP labels chromosomes, does not disrupt the cell cycle, alter the doubling time or produce any defects previously unseen in fixed cells.

A,C,E: DAPI stained non-transfected cells.

B,D,F: H2B-GFPexpressing cells.

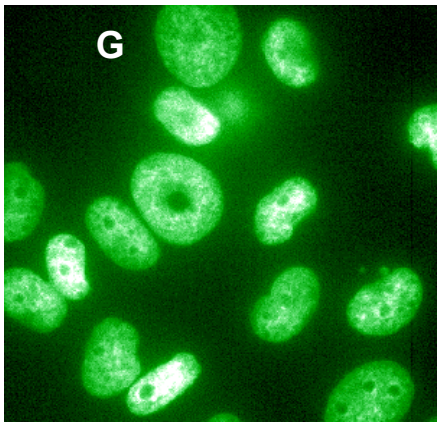
A , B: normal metaphase configurations,

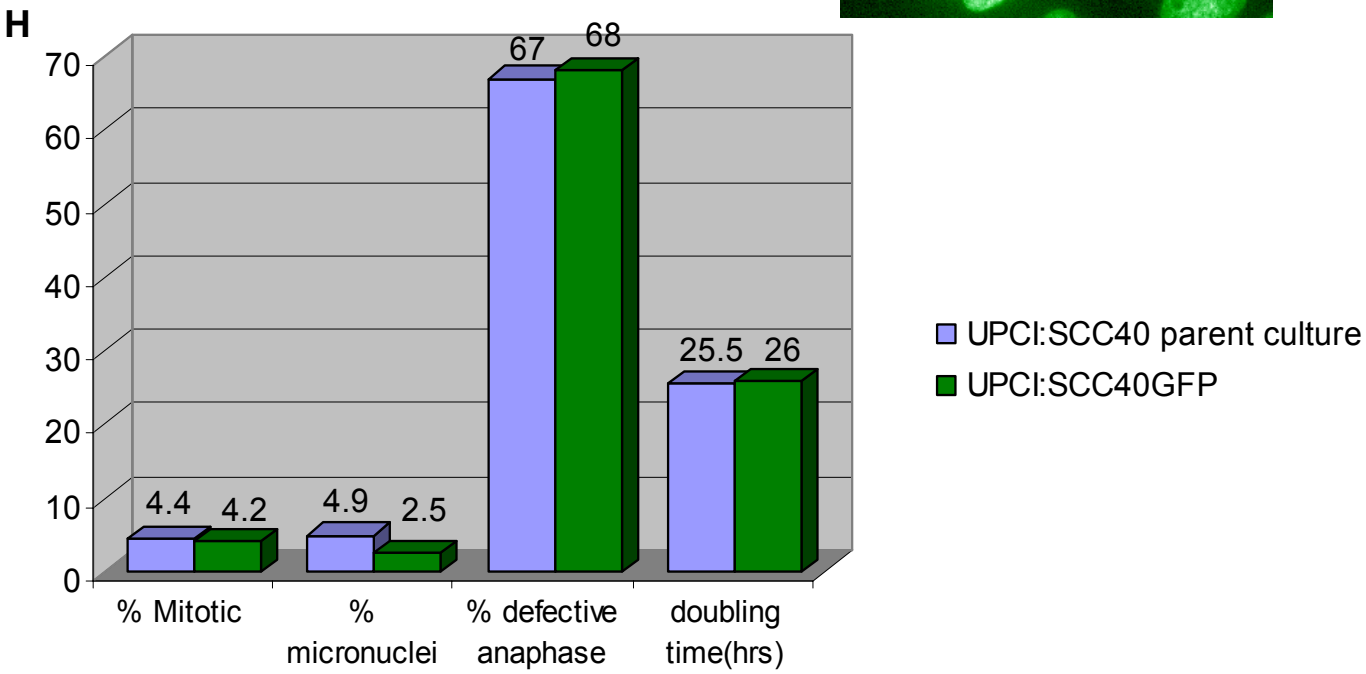
C, D: multipolar metaphases,

E, F: micronuclei in interphase,

G: approximately 75% of cells in culture express H2B-GFP after selection with blasticidin S.

H: comparison of defects and doubling time in H2B-GFP expressing cells and non expressing cells.





3.3 Segregation defects do not block cell division in cancer cells.

Next, we used this reagent to determine the fate of cancer cells with these types of segregation defects. Although clear, detailed images of chromosome dynamics could be obtained when acquiring images with relatively long light exposures and high magnification, many experiments resulted in no cell divisions and little cell motility in GFP expressing cells. Cellular damage may have occurred when imaging the cells as the environmental conditions were not optimal in early experiments. Excitation of the GFP fluorophore contained within the chromatin may have resulted in DNA damage. We then optimized image acquisition to lessen any possible photo-toxicity (described in detail in materials and methods section). Using these optimized conditions, both GFP expressing cells and GFP negative cells appeared similar in their movement and division frequency. Long-term time-lapse movies were obtained under these conditions with reduced image quality but preventing, as much as possible, any damage to the cells.

Time-lapse images of a normal division appear in movie 1. (Movies can be viewed on a QuickTime player by clicking on their title. The scroll bar at the bottom of the player can be used to view individual images. The size of the images can be increased by choosing 'movie' on the toolbar). This normal appearing division that begins with an interphase cell was acquired at 40X magnification at 2 minute intervals. Chromosome condensation and congression can be seen as the cell quickly proceeds into prometaphase and metaphase. Anaphase then occurs without any lagging or bridging chromatin. Of the 45 observed divisions that were in focus throughout mitosis, 17 appeared normal.

Two examples of anaphase bridges can be seen in movie 2 and movie 3. These images were acquired with a 100X oil objective at 30 second intervals. The chromatin bridges can be seen from the onset of anaphase. The bridges break without blocking the separation of segregating chromosomes. In order to determine whether bridges lead to cell death, we followed 26 cells dividing with bridges. Only one of the 26 cells that contained spontaneous anaphase bridges underwent apoptosis before the next mitosis began. Apoptosis was seen as chromatin fragmentation and blebbing of the cell's cytoplasm. When examined with live cell imaging, less than 1% of all cells underwent apoptotic cell death. All of the cells that contained micronuclei that were followed to the next division, were able to divide again. These observations suggest

that these two defects do not force cells to undergo apoptosis and do not appear to effect the short term viability of cells.

We also observed a multipolar division that proceeded through mitosis without arresting (movie 4). This cell appeared to form four spindle poles and during anaphase many bridges and chromatin fragments were seen between the segregating chromosomes. Three or four daughter cells were produced (a possible fourth cell was seen to descend into a lower focal plane). The smaller daughter cells appeared to undergo apoptosis as seen with simultaneous DIC imaging. The two larger progeny of the multipolar division survived and were still viable when imaging ended 16 hours later. Progeny of a multipolar division would clearly be aneuploid.

Next, we examined whether cells with segregational defects produce progeny that contain the same defects when they divide. Complete cell cycles were observed in the progeny of fifteen cells although most were not in focus for both the parental and progeny divisions (time-lapse movies were acquired over 24 hours with computer-driven auto-focus). The few in-focus divisions suggested that defects were not always seen in consecutive generations. For example, a normal appearing parental divisions produced daughters with defects. A cell that divided normally (movie 1) produced two daughter cells that divided 20 hours later, each with similar defective anaphases (movie 5). During anaphase, similar lagging and fragmenting chromatin can be seen in each daughter cell. This suggests that the segregation defects were produced in the daughter cells by similar mechanisms although the parent cell did not contain any visible segregational defects. While these results are intriguing, additional sampling will be required for complete characterization.

4. Discussion:

With live cell imaging, we have shown that mitotic segregation defects are not simply evidence of arrested and dying cells. Cells were able to divide with defects and continued to be motile and divide again in relatively normal cycle times. Anaphase bridges did not halt anaphase separation. Micronuclei did not block or appear to interfere with division. We found that it is possible for a multipolar metaphase cell to proceed through mitosis in one example. This cell did not correct the multipolar spindle by reverting to a bipolar spindle but continued to divide into more than two daughter cells with clearly unbalanced segregation. The majority of cells dividing with defects were able to divide again and can thus effectively contribute to further

generations of cells. Survival of these cells to the next division allows them to potentially contribute to tumorigenesis.

We have shown that segregational defects are found in all examined cell lines and cultures. The frequencies of specific defects vary between cell lines but these frequencies do not change significantly through multiple generations implying that cell culturing does not induce further defects and that defects may be heritable. Defects were seen in non-cancerous primary cultures of keratinocytes. These defects may be evidence of random errors in segregation or evidence of damage-induced defects as some of the patients were smokers. If this is a true reflection of how often spontaneous defects occur in normal tissue, then checkpoints may be very effective in keeping defective cells from proliferating. We propose that DNA damage or damage to the mitotic machinery from tobacco use may produce segregation defects which then lead to chromosomal instability. In future experiments we will induce segregation defects in normal cells in culture and then observe the fate of these cells in order to determine whether they can induce and perpetuate chromosomal instability in cells with intact checkpoints.

CHAPTER III. Anaphase bridges can lead to the formation of micronuclei, which represent a loss of functional genetic expression and a contribution to chromosomal instability.

1. Introduction:

We have shown that the progeny of divisions containing anaphase bridges are viable, so we now address the consequences of these bridges. Anaphase bridges are most often thought to be evidence of dicentric chromosomes. A mechanism for the resolution of dicentric chromosomes, the breakage-fusion-bridge (BFB) cycle, was proposed by B. McClintock 1941) Dicentric chromosomes have been observed in the karyotypes of many kinds of cancer cells. It has been proposed that the BFB cycle is the main mechanism of producing dicentric chromosomes, anaphase bridges, and amplification of mammalian DNA (Moore et al., 2000). This model could explain the variability seen in chromosomal karyotypes, as each division would produce new chromosomal arrangements. The initial dicentric chromosome may be produced by a double strand break or telomeric fusion. Once a dicentric chromosome exists each division may produce new chromosomal arrangements. The BFB model described in figure 4 shows dicentric sister chromatids, each attached to opposite poles. During segregation, the dicentric chromosomes may be pulled between opposing poles and may eventually break somewhere between the kinetochores. Soon after division, the chromosomal fragments' broken ends are proposed to rejoin or as shown, may rearrange and fuse with other broken chromosomes. If this fusion doesn't occur before replication, the chromosomal fragment may replicate and repair mechanisms may lead to the fusion of the two ends that do not contain telomeres, thus forming another dicentric chromosome. This breakage could lead to both copies of a gene remaining attached to one centromere resulting in the amplification of this gene. It has been shown in *Saccharomyces cerevisiae* that the joints between regions of amplified DNA is located between the centromere and telomere suggesting that breakage and subsequent fusion is occurring on telomere-less fragments thus producing dicentric chromosomes (Moore et al., 2000). These dicentrics can then repeat the cycle; bridging, breaking, and fusing during the next division. Variations of these models have been widely proposed but there is little direct evidence supporting them in mammalian cells. If anaphase bridges are products of dicentric chromosomes, we expect to see a correlation between the frequency of bridges and dicentric chromosomes.

When analyzing the fate of cells dividing with anaphase bridges, we saw the formation of micronuclei. The existence of micronuclei has been used as a way to measure chromosome damage for several decades (reviewed by Fenech, 1997). It is generally assumed that micronuclei are a consequence of chromosome breakage and loss but the mechanism of generating micronuclei is basically unknown. Furthermore, it is unclear if the DNA contained within micronuclei is transcriptionally active.

We used live cell imaging, indirect immunofluorescence, and an adaptation of transcriptional assays in order to further analyze the consequences of bridge formation. We were unable to determine what happens to specific chromosomes with our current labeling system, but we were able to determine that bridges, in general, resolve by breakage and subsequent formation of micronuclei. These micronuclei are not predicted by the BFB cycle and complicate the analysis of gene and chromosomal amplification. Fragments of each bridge detach from the rest of the chromosome and thus are not involved in the next cycle of possible dicentric bridging. We also determined that micronuclei are not transcriptionally active and so these fragments do not function in gene expression and do contribute to chromosomal instability.

2. Materials and Methods:

2.1 Live cell imaging experiments and indirect immunofluorescence

as previously described

2.2 Transcription assay:

Chromatide AlexaFluor 488-5-UTP was purchased from Molecular Probes and α-amanitin was purchased from Sigma. UPCI:SCC40, UPCI:SCC103 and diploid fibroblasts (GM03349B Coriell) cultures were grown on coverslips in M10 medium with 25mM HEPES for 24 hours. Medium was removed and 50 µl of HEPES M10 was added containing either 0, 50 or 500µg/ml α-amanitin. After 30 minutes incubation at 37^0C 5%CO_2, 20µl of 0.05mg/ml AlexaUTP was added, 100µm glass beads (Sigma) were sprinkled onto the cells to permeabilize the plasma membrane, coverslips were then either washed in MEM (Gibco BRL) after 3 minutes to remove the beads and unincorporated analog or returned to the incubator without washing. Coverslips were removed at different time intervals and live cells were viewed for fluorescence or fixed with ethanol, stained with DAPI and viewed for both FITC and DAPI.

3. Results:

3.1. Anaphase bridges lead to the formation of micronuclei.

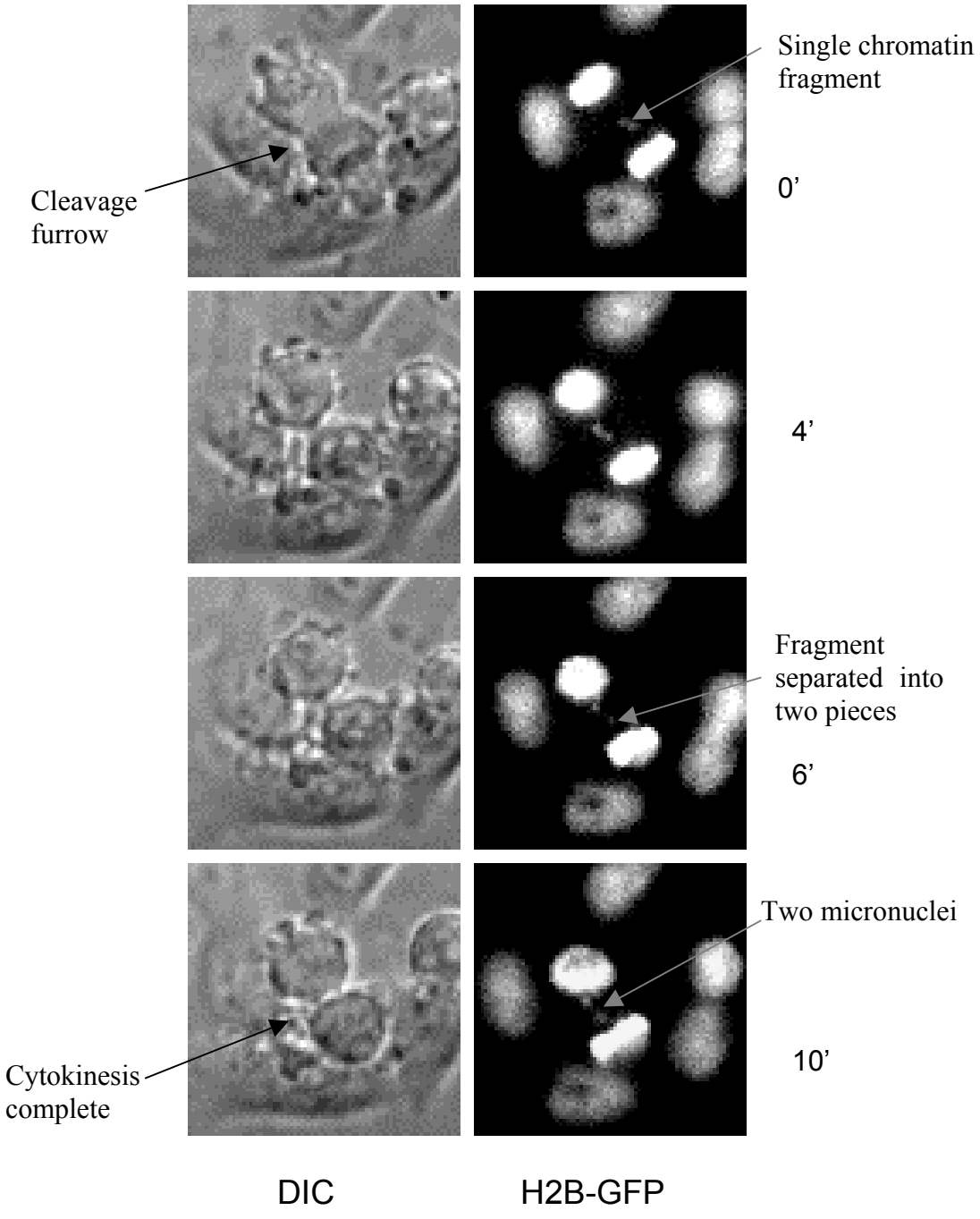
To determine the fate of cells dividing with anaphase bridges we again used live cell imaging of the cells labeled by H2B-GFP expression. We observed that chromatin bridges quickly break during anaphase. The breaks often do not appear to occur directly at the midpoint of the bridge. The resulting fragments formed micronuclei. The complete cell division shown in movie 6 is an example of a bridge resulting in the formation of a micronucleus. The chromatin in the bridge appears to have broken at more than one site to form this separate fragment. At least one micronucleus was produced in all 24 divisions observed that contained anaphase bridges where focus was maintained throughout division. None of these cells contained micronuclei before division. Often, each daughter cell contained a micronucleus. Movie 7 contains images of an anaphase bridge breaking and a micronucleus forming. The chromatin strand can be seen breaking and then appears to recoil into the segregating chromosomes suggesting that the chromatin is under tension. A micronucleus forms at the end of mitosis and is seen later in interphase. In one example, cytokinesis was coincident with breaking of the fragment and may have contributed to breakage (figure 8). In this time-lapse series, a chromatin fragment is broken into two micronuclei. One micronucleus remains with each daughter cell. When examining DIC images taken simultaneously with the GFP images, the cleavage furrow appears to separate the chromosomal fragment into two. In summary, we have shown that anaphase bridges resolve by breaking at multiple sites and forming micronuclei, thus possibly contributing to chromosomal instability.

Next, we attempted to determine the fate of micronuclei. The movement of micronuclei was very dynamic with respect to the main nucleus and at times the micronuclei were hidden from view. Movies 8 and 9 contain examples of the movement of a micronucleus. The micronucleus can be seen moving away from the main nucleus and then returning soon before mitosis in movie 8. Movie 9 contains a montage of both GFP and DIC images of the same cell (now in the upper left corner of the field). It appears that the micronucleus is not tethered to the main nucleus as it moves as much as three nuclear lengths away from the main nucleus, traveling down into a lamellipodia and then rebounding back shortly before mitosis. The micronucleus is lost from view during anaphase. It appears that the micronucleus may be left between the segregating daughter cells although the cells leave the focal plane and this cannot be confirmed.

The montage shows that the majority of cells are labeled with H2B-GFP but two unlabeled cells can be seen dividing in the DIC images in the center of the field near the end of the movie. The DIC images also make it clear that these cancer cells are quite motile. When observing micronuclei for complete cell cycles, they were never seen integrating into the main nucleus during interphase. In the few examples observed where a micronucleus is followed before and during mitosis, it was not possible to determine whether the micronuclei integrated into the main nucleus.

Figure 8: Cytokinesis coincident with micronuclei breakage.

A UPCI:SCC40 cell expressing H2B-GFP with a chromosomal fragment remaining between the separating chromosomes. DIC images show cytokinesis occurred between 4 and 6 minutes. The fragment was separated into two pieces at approximately the same time.



3.2 Micronuclei are not transcriptionally active.

In order to address the question of whether micronucleation is an actual loss of coding DNA, we preformed a transcriptional assay using both oral cancer and fibroblast cells. If the chromatin found in micronuclei continues to function in gene expression then formation of micronuclei may have a minimal impact on the cell. We chose to begin examining whether the DNA in micronuclei is functional in gene expression by examining transcription, the first step in gene expression. Visualizing sites of transcription has previously been accomplished by the incorporation of [^{3}H]UTP, the microinjection of BrUTP into individual cells, or by the incorporation of BrUTP into encapsulated, permeabilized cells and then fluorescent or autoradiographic detection of these labels (Wansink, Schul et al. 1993; Cmarko, Verschure et al. 2000). To label random cells containing micronuclei it was necessary to label many cells at once. We chose to adapt a simpler and less disruptive method that has been used previously to label DNA in living cells by the incorporation of a fluorescent nucleotide to analyze the formation of chromosomes and their movement in Indian muntjac cells (Manders, Kimura et al. 1999). The nucleotide was introduced into the cells by glass bead loading (McNeil 1989). Glass bead loading is a rapid method of loading molecules into the cytoplasm of cells by disrupting the plasma membrane. The beads temporarily permeabilize the plasma membrane to allow the fluorescent analog into the cells where it can then be transported into the nucleus. This method allows the cells to remain adherent and imaging can be done immediately. In order to determine whether micronuclei are transcriptionally active, the ChromaTide nucleotide AlexaFluor 488-5-UTP (Molecular Probes) was used to visualize where and when the uridine triphosphate was incorporated into nascent RNA. At 5 minutes after addition of AlexaUTP to the cells, very little signal was seen in the cell nucleus. By 20 minutes, the nuclei of many cells contained diffuse labeling. After one hour, signal was seen as punctuate dots in the cytoplasm of the cells as might be seen if nascent RNA was transporting out of the nucleus (figure 9).

Micronuclei were not labeled by incorporation of AlexaUTP in oral cancer cells (figure10). This suggests that the DNA contained in micronuclei is not transcriptionally active. Large fragments of chromatin and blebs that appear to be still attached or situated very close to the main nucleus are labeled and transcriptionally active (figure 10). Thus, any genes trapped within micronuclei do not appear to be expressed.

To distinguish between transcriptional incorporation of the labeled UTP and simple transport into the nucleus, the cells were treated with α-amanitin which specifically and selectively inhibits transcription by RNA polymerase II but not PolI and PolIII (Masson 1996). In the presence of inhibitor, the UTP signal changed from diffuse nuclear to perinucleolar in both fibroblasts and oral cancer cells (figure 11). This residual perinucleolar staining is presumably due to continued transcriptional activity by PolI and PolIII. This pattern of signals was also seen when BrUTP was used to examine localization of transcription (Hassan 1994; Masson 1996).

Figure 9: AlexaUTP signal after UTP incorporation.
Typical images of fibroblast and oral cancer cells after UTP incorporation.

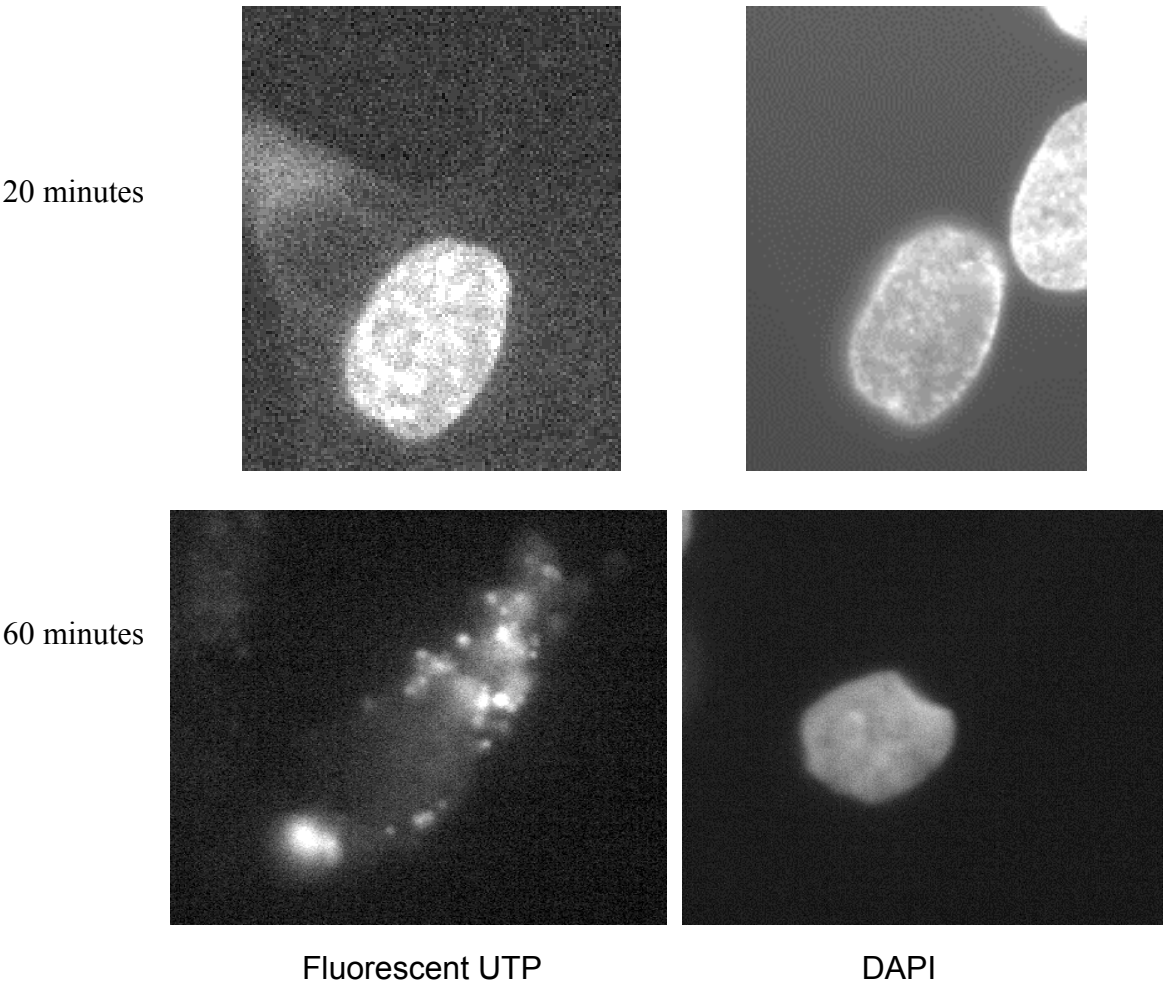


Figure 10: Fluorescent UTP incorporation.

UPCI:SCC103 cells were loaded with AlexaFluor 488-5-UTP, incubated 20 minutes, and stained with DAPI to visualize DNA . These representative cells contain transcriptionally inactive micronuclei (arrows) and transcriptionally active blebs (arrowhead).

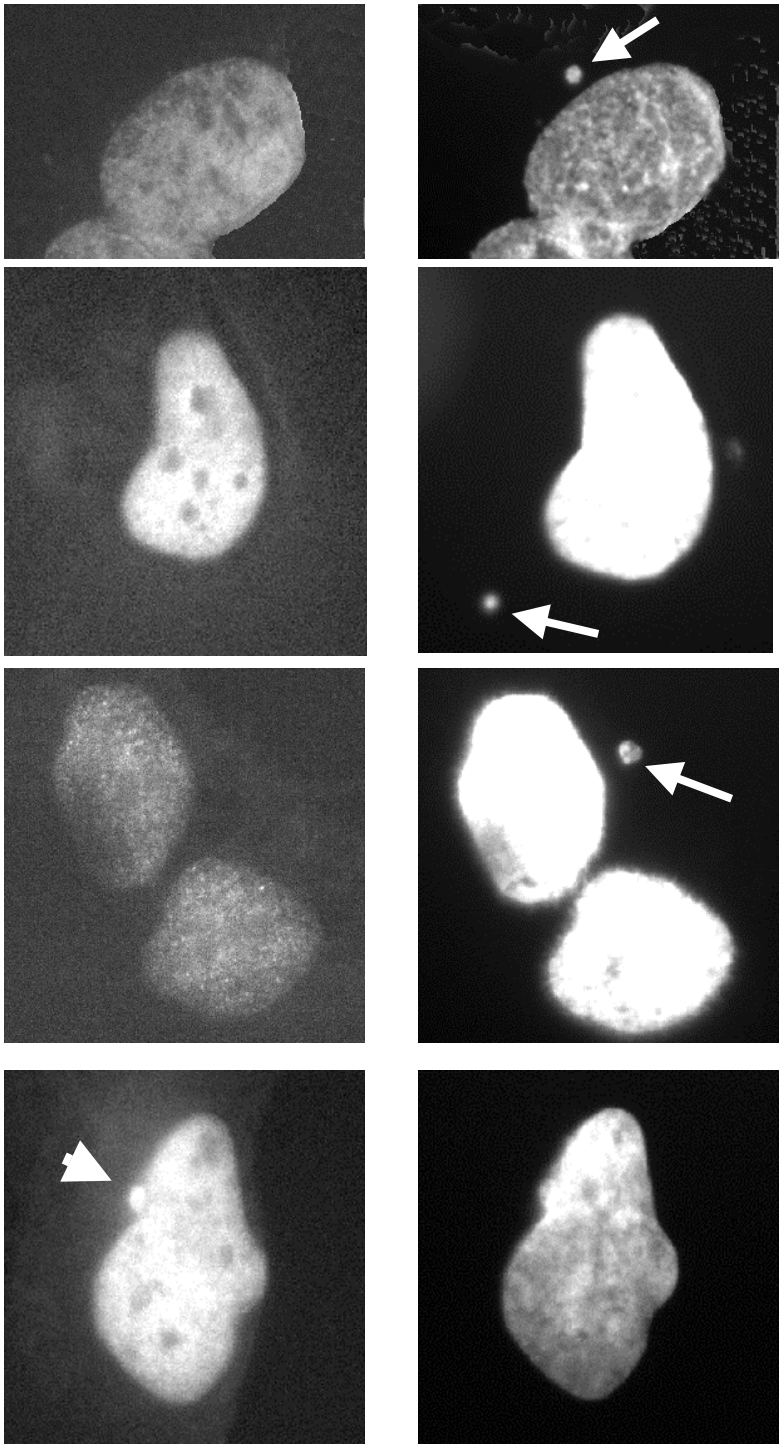
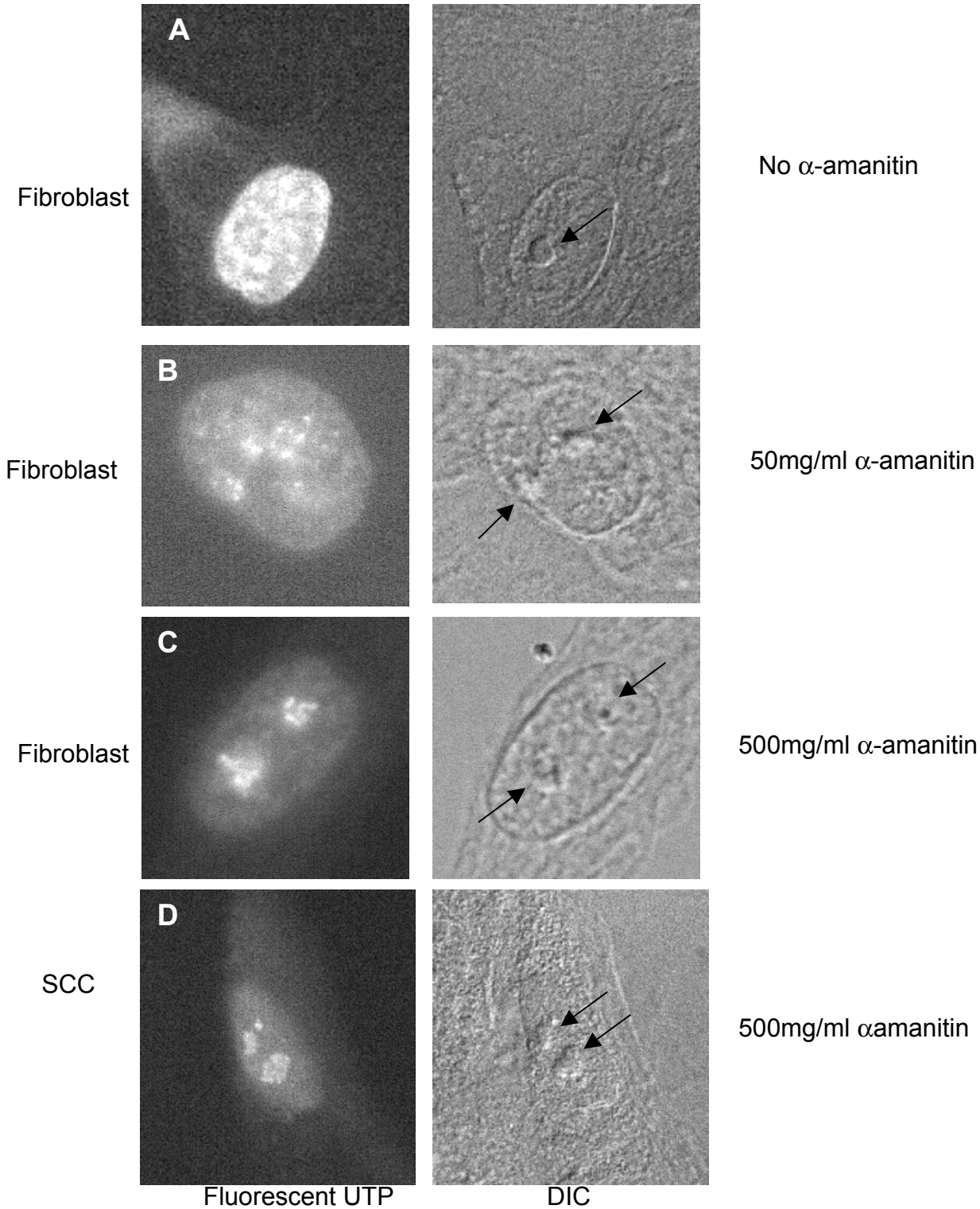


Figure 11: Incorporation of fluorescent UTP (AlexaFluor 488-5-UTP) is specific to transcription. Cells were incubated in either 50 or 500mg/ml α-amanitin before and during UTP loading. α-amanitin specifically and selectively blocks PolII allowing continued transcription by PolI and PolIII localized near the nucleolus. DIC images are shown to visualize nucleoli (arrows).



3.3. Anaphase bridges may also be resolved by the formation of interphase bridges.

Chromatin bridges were seen in fixed interphase cells when viewed with DAPI. These interphase bridges may be unresolved anaphase bridges. The cell lines containing the highest levels of anaphase bridges also contained the highest levels of interphase bridges, for example UPCI:SCC103 had 68% anaphase bridges with 9% interphase bridges while UPCI:SCC70 had 16% anaphase bridges and only 1% interphase bridges. This suggests that anaphase bridges could lead to the formation of interphase bridges when they do not break and form micronuclei. Interphase bridges were not observed in live cell imaging most likely because the optical conditions required to maintain cell viability did not permit the visualization of these small structures.

4. Discussion:

We have shown that chromatin bridges between segregating sets of chromosomes will break and result in the formation of micronuclei. For anaphase bridges to form micronuclei, the strand must have broken in more than one location to obtain three or more fragments from one strand. In a 'tug of war' with a complete strand of material, the forces generated from both ends will break the strand in only one place. When two micronuclei form, the strand must have broken in at least three places. If the only forces acting upon the strand are originating from the spindle poles, then even if the strand has several weak or fragile sites, there would be no more forces to act upon the other sites once the first break has occurred. It appears that other forces are acting upon the stretching strand. As seen in figure 8, cytokinesis may have an effect on breaking the chromatin. A fragment may be severed when it is trapped in the cleavage furrow or the tightening furrow may hold the midsection of the strand firmly, providing a third point of attachment. The strand may then break between each pole and the midbody. As micronuclei are being formed by more than one break in each chromatin bridge, uneven segregation of chromosomes between daughter cells results thus leading to chromosomal instability.

Evidence of the integration of micronuclei back into the main nucleus was not observed during interphase, but I was unable to follow these fragments effectively during the critical period of nuclear envelope breakdown during mitosis. I have also shown that the gene expression from DNA fragments within micronuclei is lost. Large fragments and blebs probably

remain transcriptionally active because the transcriptional machinery still has access to these fragments. Thus, gene expression from these distinct micronuclei is lost. It is currently unclear if the cell regains expression of the DNA contained within micronuclei during the next cycle.

Previously, it was assumed that if micronuclei contained centromeres, then that DNA fragment is actually a whole chromosome and loss of this complete chromosome (Schuler, Rupa et al. 1997). As I have shown that micronuclei formation occurs by breaking of chromosomes into small fragments and not just from whole chromosome loss, the presence or absence of centromeres probably does not correspond to the size of the chromatin within the micronuclei. The presence of centromeres may simply be a reflection of the randomness of breakage (figure 3D). The largest effect of centromeres contained within micronuclei may be that they increase the possibility for integration back into the main nucleus during the next division. This would probably minimize the effect of some micronuclei on gene expression to only one cell cycle. But micronuclei do appear to have an impact on chromosomal stability for at least one cell cycle.

Chromatin bridges have also been seen during interphase. The anaphase bridge may not have been broken in these cells and the bridge then persists into interphase. In fixed cell analysis, cell lines containing higher levels of anaphase bridges also contain higher levels of interphase bridges. It is unclear whether these cells have arrested once an interphase bridge forms. The consequences and significance of interphase bridges are unknown.

If anaphase bridges are due to dicentric chromosomes, then a correlation should be seen between the frequency of bridges and dicentrics. Our collaborators have shown by metaphase analysis with C-banding that an average of 7% of chromosomes in each UPCI:SCC40 cell are dicentric. More than 96% of cells (n=26) contained one or more dicentric chromosome (Camille Rose and Susanne Gollin, unpublished results). With live cell imaging, we have observed that approximately 58% of anaphase cells in UPCI:SCC40 contain bridges and rarely is more than one bridge seen in each cell. Although we saw a high frequency of bridges we would expect to see bridges in virtually all divisions based on the frequency of dicentrics. The source of this discrepancy may be explained by not all dicentrics forming bridges in each division. A bridge in every division is not necessarily predicted by the BFB model. Only when both centromeres of a dicentric chromosome are attached to opposite poles will a bridge form, thus, fewer bridges than dicentrics may be seen. Also, a dicentric may not form a bridge if it contains one inactive centromere and thus attaches in a normal bipolar manner. Both centromeres may also attach to

the same pole. But if monopolar attachment occurs in these cells, then the cells must be immune to the spindle checkpoint, as monopolar attachment would not produce tension on the kinetochore. As this predicts a possible loss of the spindle checkpoint, we next examined whether checkpoints are active in these oral cancer cells. In summary, this evidence of breakage, dicentric chromosomes, micronuclei formation and loss of gene expression in surviving cells suggests that chromosomal instability caused by segregational defects is an ongoing process in these cancer cells.

CHAPTER IV. Oral cancer cells have at least a partially intact spindle checkpoint that causes delays during division but defective cells are able to undergo a normal anaphase.

1 Introduction:

The mitotic spindle checkpoint prevents cells from exiting mitosis when there is a chance that improper segregation may occur. Two situations may activate the spindle checkpoint; either the lack of microtubule attachment to kinetochores or the lack of microtubule tension on the kinetochores (reviewed by (Amon 1999). When either of these defects occurs, it has been shown that cells will arrest until the defect is corrected. Sister chromatid separation is initiated by the APC degradation machinery and this machinery is inhibited when the spindle checkpoint is activated. It is possible that mutations in any of the components of the spindle checkpoint pathways (including the MAD/BUB genes) may inactivate the spindle checkpoint. It has been proposed that the loss of a functioning spindle checkpoint mechanism is responsible for aneuploidy in colorectal cancers and that most cancer cells have inactive checkpoint mechanisms (Cahill, Kinzler et al. 1999). When they expressed BUB mutants in normal cells, the cells did not arrest at mitosis when the spindle was damaged. A defective checkpoint mechanism may be what allows cells to proliferate with defects and thus produce chromosomal instability. We are interested in determining whether these oral cancer cells have an intact spindle checkpoint and whether this checkpoint is detecting the errors we are seeing.

In order to determine whether the oral cancer cells have intact checkpoint mechanisms we analyzed the timing of each phase of mitosis and compared these times with cells dividing

with different types of defects. Using live cell imaging we were able to distinguish between the different phases mitosis and used these time points to establish the length of each phase. We find that anaphase bridges and lagging metaphase chromosomes delay the onset of anaphase in these oral cancer cells. This would be expected if the spindle checkpoint is intact. But some cells with defects are still able to exit from mitosis which is not expected if the spindle checkpoint is fully functional. It appears that the checkpoint mechanism is detecting the defects but not causing complete cell cycle arrest, allowing perpetuation of chromosomal instability.

2. Materials and methods:

2.1 UPCI:SCC40 H2B-GFP expressing cells were examined with live cell imaging as previously described, data acquisition and analysis was achieved using Metamorph imaging software (Universal Imaging Corp., Downingtown PA). Statistical significance and standard deviations were determined by Students T test analysis.

3. Results:

3.1 Metaphase is lengthened in cells containing anaphase bridges and lagging metaphase chromosomes.

To analyze whether spindle checkpoints are intact in oral cancer cells we determined the length of each phase of mitosis using live cell imaging. Timing of prophase began with chromosome condensation and rounding of the cell seen by simultaneous DIC images. Metaphase could be seen as chromosome congression to the metaphase plate and the first image of separation of the segregating sets of chromosomes was determined to be the onset of anaphase. Chromosome decondensation signaled the exit from mitosis. A cell division was labeled 'normal' if no lagging chromosomes or bridges were present during that division.

The average length of time from prophase to the onset of anaphase is shown in figure 12. On average, 43.3 minutes elapsed between prophase and anaphase for normal divisions in these cancer cells. Chromosomes that lagged behind the majority of congressing chromosomes significantly delayed the onset of anaphase by an average of 19 minutes ($p=0.005$ with $p< 0.05$ being statistically significant). We observed one example of a division with lagging metaphase chromosomes that did not reach the metaphase plate before anaphase onset. The cell delayed

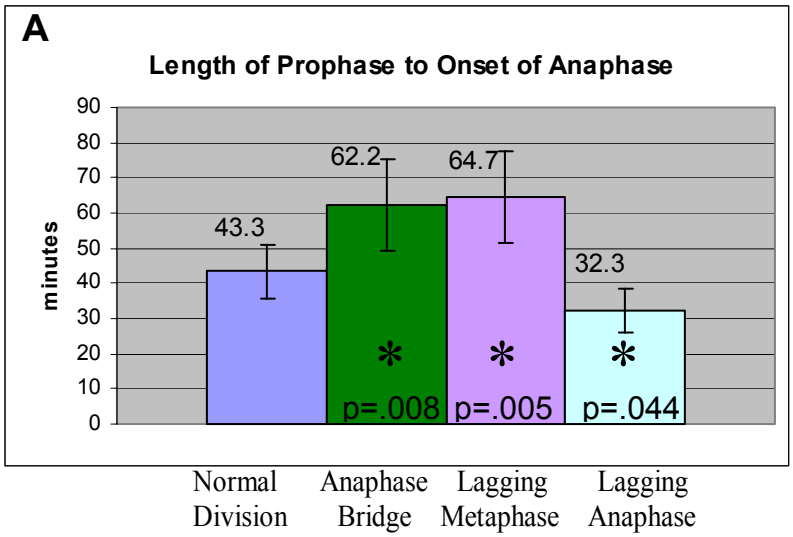
anaphase, presumably because the spindle checkpoint was activated, but then entered anaphase without correcting the defect. This should not happen if the checkpoint is intact but we cannot rule out an interfering effect from imaging the cells. No other divisions were observed where anaphase began without all the chromosomes first reaching the metaphase plate. We conclude that the spindle checkpoint is at least partially intact in these cells.

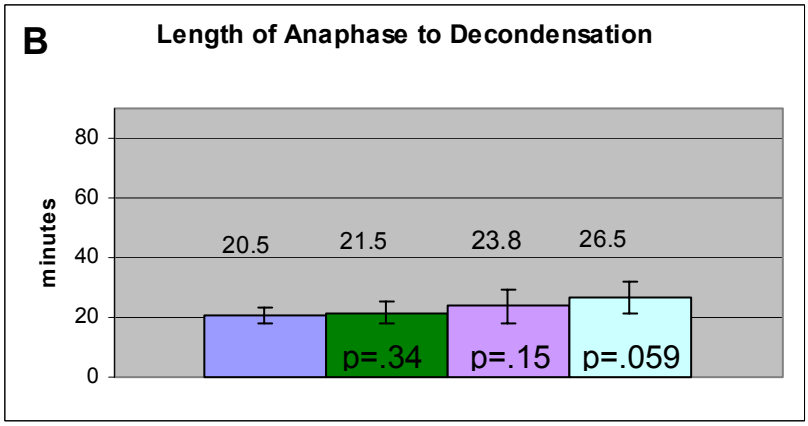
Anaphase bridges significantly delayed the onset of anaphase (figure 12A, p= 0.008). Anaphase bridges cannot be seen before the onset of anaphase but the delay suggests that some type of checkpoint had been activated. This delaying mechanism may be a new, previously uncharacterized checkpoint or may be a new function of the already characterized spindle checkpoint. Most divisions containing anaphase bridges did not previously contain lagging metaphase chromosomes so the delay from bridges is not due to lagging metaphase chromosomes. It is currently unknown if the new, putative checkpoint is distinct from the spindle checkpoint.

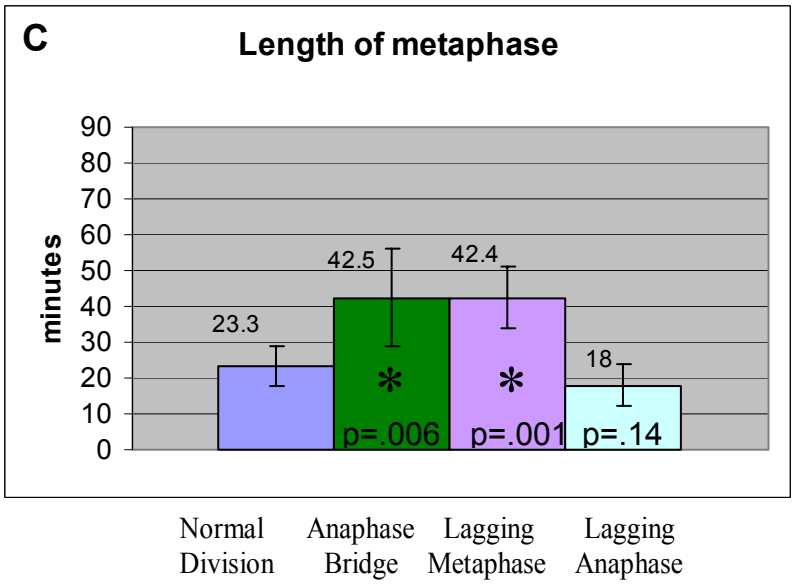
We next asked whether all defects lead to delays in anaphase onset. We find that lagging chromosomes seen between segregating chromosomes during anaphase actually corresponded to an acceleration of anaphase onset (figure 12A, p= 0.044). These changes in cell cycle timing could reflect physical or regulatory effects. To test whether the delays or acceleration are dependent on the stage of mitosis, we also tested the timing of anaphase in cells with segregation defects. None of the defects significantly slowed the completion of anaphase (figure 12B). Thus, either for mechanical or regulatory reasons these delays are limited to pre-anaphase.

3.2 Mitotic delays occur specifically during metaphase

In order to determine if the cell cycle delays were occurring during metaphase as would be seen if the spindle checkpoint was active, we examined the differences in time between chromosome condensation, chromosome congression and the onset of anaphase. The length of prophase was approximately the same in all types of defective and normal divisions. The average lengths of metaphase are shown in figure 12C. Metaphase was significantly longer in cells containing anaphase bridges and lagging metaphase chromosomes (p=0.006 and p=0.001 respectively).







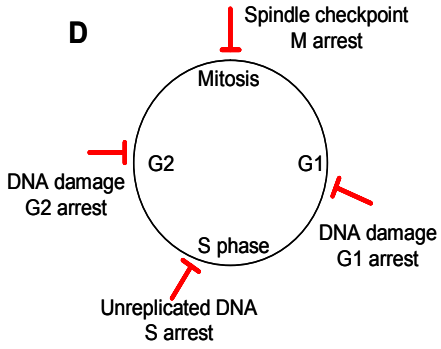


Figure 12: The consequences of segregation defects on the length of mitosis.

A: Segregational abnormalities delay the onset of anaphase as determined by timing from prophase and chromosome condensation to the onset of anaphase. Significant differences between the average length of time for each defect and the average time for normal divisions are shown by a * , student T test p values are shown for each defect. Sample size is an average of 15 different cells for each defect.

B: Segregational abnormalities do not delay anaphase after separation has occurred. No significant differences were observed between normal division and defective divisions

C: Segregational abnormalities lengthen the time spent in metaphase, anaphase bridging and lagging metaphase chromosomes lengthen metaphase significantly.

D: Known checkpoints effecting the cell cycle (adapted from Darnell et al., 1995).

3.3 The length of mitosis is longer and more variable in cancer cells when compared to normal cells.

In order to compare the length of mitosis in non-transformed cells to oral cancer cells, we transfected diploid fibroblasts with H2B-GFP. Transfection efficiency of these cells was too low to be useful and normal oral keratinocytes do not survive in culture long enough to view stable transfections. To compare the timing of mitosis we then analyzed time-lapse series of DIC images of non-transfected fibroblasts. The average length of time between cell rounding (corresponding to chromosome condensation) and anaphase onset was 20 minutes in unlabeled fibroblasts (n=6) with a range of 10 to 34 minutes. The length of mitosis in 12 cancer cells examined by DIC was 34.7 minutes which is significantly longer then fibroblasts (p=0.006). In these cancer cells the time varied from 20 to 52 minutes. The cells were chosen randomly as DIC images do not allow differentiation between segregation defects. We examined many more cancer cells labeled with H2B-GFP (n=91) and found that the length of mitosis varied between 18 to 130 minutes in labeled cells.

4. Discussion:

UPCI:SCC40 oral cancer cells appear to have an intact mitotic spindle checkpoint which was seen as delays during metaphase in cells containing defects. It appeared that the majority of cells were able to delay the onset of anaphase until all chromosomes had reached the metaphase plate. This delay was observed when lagging chromosomes were present, suggesting that the checkpoint mechanism for monitoring proper bipolar kinetochore attachment is intact. One example of an anaphase occurring before a lagging chromosome reached the metaphase plate was seen in our lab with a new microscope and imaging system. This cell may have actually been a multipolar cell with the lagging chromosome attached to a third spindle pole. We will do further analysis with this new equipment to determine how frequently anaphase can occur when lagging chromosomes are still present.

Metaphase delays occurring before anaphase bridges may be the result of an intact spindle checkpoint. But if this checkpoint is the mechanism of arrest, then it is not functioning as previously characterized as both tension and occupation of the kinetochores should have been satisfied. Curiously, the putative checkpoint that is effecting the division of these cells is sensing

the bridges before they actually occur. A new mechanism sensing a disruption in kinetochore tension or attachment may be activated by the presence of a dicentric chromosomes. For example, tension on two kinetochores through the length of a chromatid may register differently then tension between sister kinetochores on duplicated chromosomes. Alternatively, this new surveillance mechanism may be activated by something unrelated to kinetochore function. A third possibility may be that a physical block and not a checkpoint surveillance mechanism is disrupting division. Although, this physical block is not due to the stretching chromatin bridge as delays are not seen during anaphase but earlier in metaphase. The new mechanism may not only be sensing the potential bridge, but may also allow repair or correct the improper bipolar attachment. A checkpoint induced release of a bipolar attachment may explain our observation of reduced incidence of bridges when compared to dicentrics. In the future, we will examine the fate of normal cells containing induced bridges which may help us gain insight into how and when this surveillance mechanism may be malfunctioning in the cancer cells.

In summary, we have observed activation of mitotic arrests when segregation defects are present. We can not be sure if the checkpoints are fully functional in these cancer cells without determining how normal diploid cells behave with induced defects. We show that anaphase bridges activate a mechanism that produces a mitotic delay and this mechanism may be a novel checkpoint.

CHAPTER V: Centrosome amplification is seen in normal keratinocytes and cancer cells. Over-expression of NuMA, a centrosomal protein, in oral cancer cells does not appear to correlate to gene amplification or spindle defects.

1. Introduction:

Interest in centrosome defects as a possible contributor to tumorigenesis has increased in recent years. Centrosome amplification and defects have been observed in many types of solid tumors (Lingle, Lutz et al. 1998; Ghadimi, Sackett et al. 2000). Since centrosomes nucleate and organize the mitotic spindle and because they are involved in ensuring proper chromosome segregation, it has been hypothesized that centrosome amplification may lead to segregation defects and thus the production of aneuploidy (Lingle et al., 1998). It is not known how cancer cells tolerate or produce extra centrosomes. The cell cycle normally ensures the duplication of only one new set of centrioles. Extra centrosomes could be a result of errors in centriole replication, cytokinesis failure, fusion of cells, or the spitting of existing centrosomes to form more than two functioning microtubule organizing centers (MTOCs). It is possible that the multipolar spindles observed in cancer cells result from centrosomal amplification or defects.

Nuclear mitotic apparatus protein (NuMA) is a 238 kDa protein that is a structural component of the nucleus that changes location depending on phase of the cell cycle; during interphase it localizes to the nuclear matrix while during mitosis it is found focused at the spindles poles (Compton, Szilak et al. 1992). NuMA is essential for mitosis as the expression of deletion constructs or microinjection of antibodies caused a prometaphase block (Compton and Cleveland 1993). It is possible that NuMA functions as a nuclear scaffolding matrix during interphase (Harborth and Osborn 1999). The precise mitotic functions of NuMA are unknown but it is has been shown to bind to the minus ends of microtubules and to interact with the motor protein, dynein (reviewed by Zeng 2000). Many NuMA studies suggest that it is involved in microtubule nucleation and formation of the spindle pole during mitosis. Over-expression of this protein may lead to an accumulation of excess centrosomal material and thus may be correlated to increases in spindle defects. We have previously suggested that an excess of NuMA in cancer cells may produce defective spindle poles, a splitting of the MTOC, and possible production of the multipolar spindles seen in cancer cells (Saunders, Shuster et al. 2000).

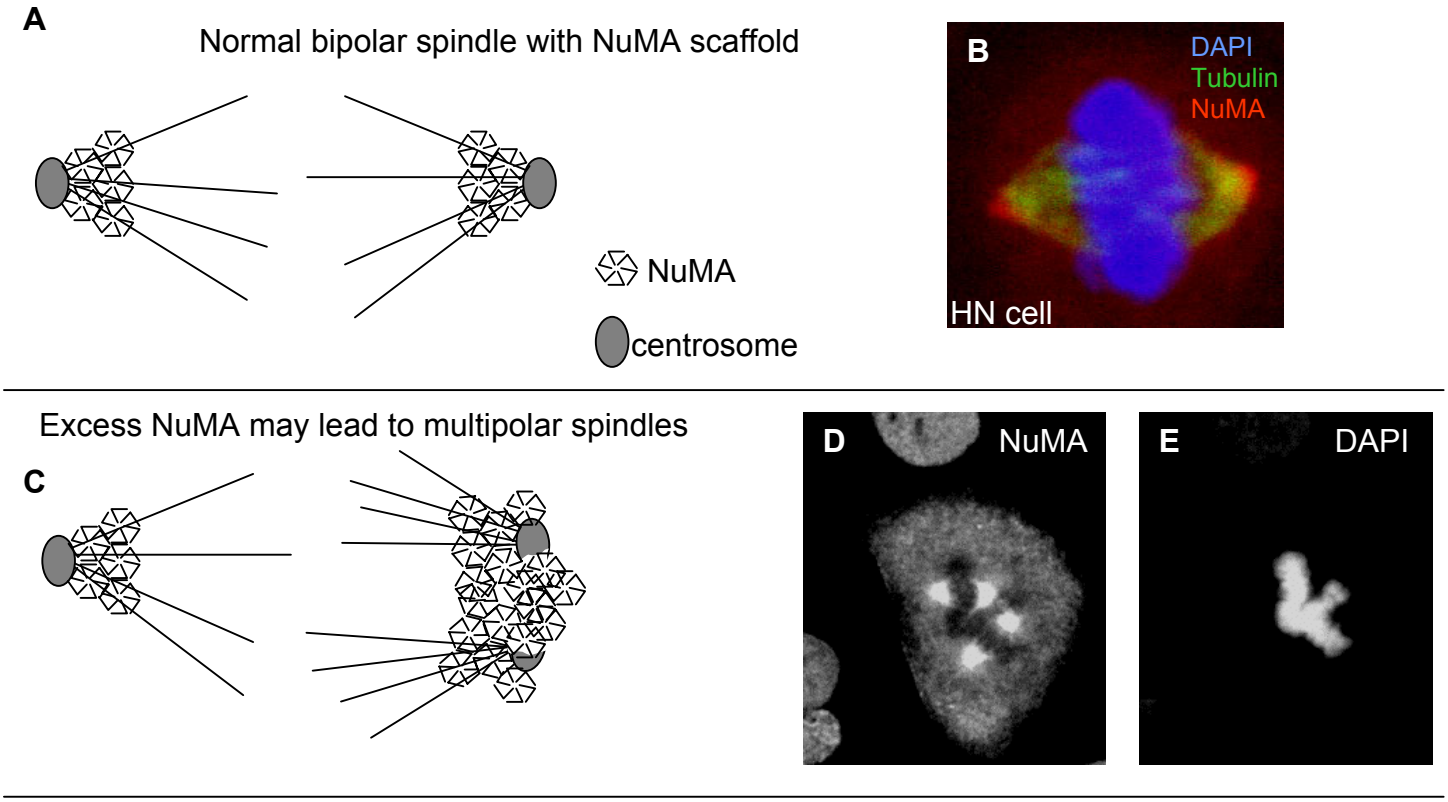
NuMA gene amplification was observed by our collaborators by quantitative PCR and fluorescent in situ hybridization (FISH) in some of the oral cancer cell lines we are examining (Xin Huang and Susanne Gollin, unpublished results). We have since examined the protein expression levels of NuMA in these same cell lines. We also analyzed the localization and expression of two other centrosomal proteins, γ-tubulin and HSET. While examining these centrosomal proteins we found unusual γ-tubulin staining with increases in centrosomal number in both normal and cancer cells. We also determined that NuMA gene amplification did not correlate to protein over-expression in all cases or to an increase in the frequencies of spindle or segregation defects. NuMA expression did vary between cell lines independently of expression of other centrosomal proteins. Increases in NuMA expression does not appear to be the only factor involved in producing segregation defects but it may have an interacting role.

Figure 13: Mitotic NuMA localization in normal and cancer cells.

A: a model of normal NuMA metaphase localization and NuMA localization during metaphase in a normal oral keratinocyte (**B**).

C: a model of the possible effects of excess NuMA on a mitotic spindle. Fragmentation and splitting of the centrosome subsequently forming of an extra MTOC may be the result of excess NuMA.

D,E: A multipolar metaphase UPCI:SCC70 cell visualized with anti-NuMA and DAPI.



2. Materials and methods:

2.1 Cell Lines:

Maintained as previously described:

UPCI:SCC 40, 70, 78, 103, 131, 142, 154, 172 oral cancer cell lines

HN-01-328, 330, 344, 348, 376 normal oral keratinocytes

2.2 Immunological techniques:

Indirect immunofluorescence: cells were prepared and stained as previously described (see Table 1 for antibodies and dilutions).

Western analysis: cells were rinsed in PBS, harvested in RIPA buffer with protease inhibitors, and stored at -20^0 C. Proteins were then separated by SDS-PAGE and transferred onto PVDF membranes by semi-dry transfer. The blots were incubated with primary antibodies (see Table 1) diluted in TBST/ 5% non-fat dry milk. Bound antibodies were detected with either anti-rabbit or anti-mouse IgG peroxidase-linked ECL conjugate (Amersham Pharmacia). Films were digitally scanned and quantified.

3. Results:

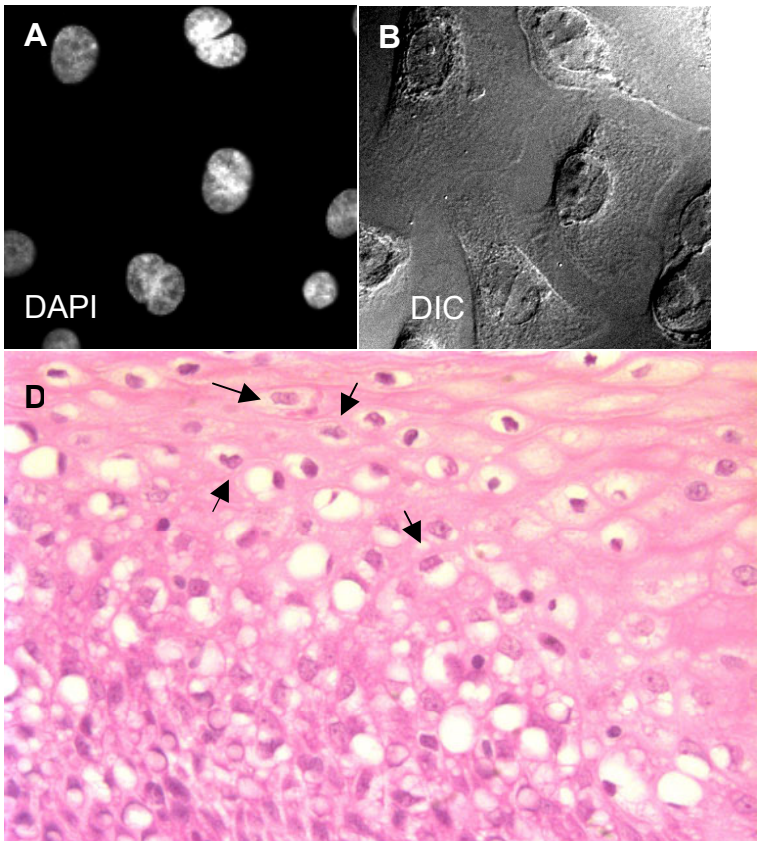
3.1 Centrosomal abnormalities found in both normal and cancer cells

We began our analysis of possible centrosomal defects in normal keratinocytes and oral cancer cells with indirect immunofluorescence using anti-γ-tubulin antibody. γ-tubulin is a non-polymerizing form of tubulin that is a component of the pericentrosomal matrix. It is found to localize at all MTOCs (Darnell et al., 1995). While examining oral keratinocytes from five patients, 2 smokers and 3 non-smokers, we noticed that a high frequency of cells contained two nuclei (figure 14 A,B). These binucleate cells were not an artifact of the specialized medium used for keratinocytes as when both fibroblasts and cancer cells were grown in this medium, no changes in the frequencies of binucleate cells were seen. In order to determine whether these binucleate cells were also found in tissue, we examined H & E stained tissue sections obtained from the same patients at the time of surgery (provided by Dr. Jennifer Hunt, Oral Cancer Center, UPMC). The tissue sections also appeared to contain binucleate cells although the

frequency is difficult to establish accurately in tissue (figure14D). Cancer cells are occasionally binucleate but normal diploid fibroblasts rarely are.

Abnormal centrosome numbers were also seen at varying frequencies when examining the normal keratinocytes with γ-tubulin staining (figure 15). Oral cancer cells also contained excess centrosomes. Most frequently, in normal keratinocytes, excess centrosomes were seen to cluster between the two nuclei with as many as 8 signals present in one cell (figure 14C). In cancer cells, abnormal centrosome numbers were also seen but the γ-tubulin signals were more often scattered around the periphery of single nuclei. These preliminary comparisons between cells derived from smokers, non-smokers, and tumors have prompted us to further examine the relationships between binucleation, excess centrosomes and smoking.

Figure 14: **A,B**; binucleate oral keratinocytes visualized by DAPI staining and DIC imaging **C**; binucleate oral keratinocyte with 6 to 8 γ tubulin signals. **D**; Hematoxylin and eosin stained epithelium section from HN-01 330 with possible binucleate cells (arrows).



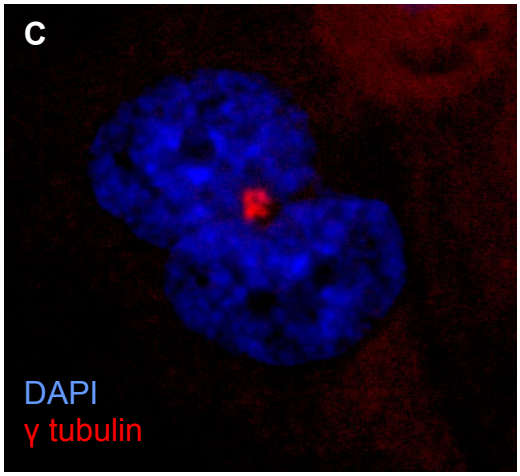
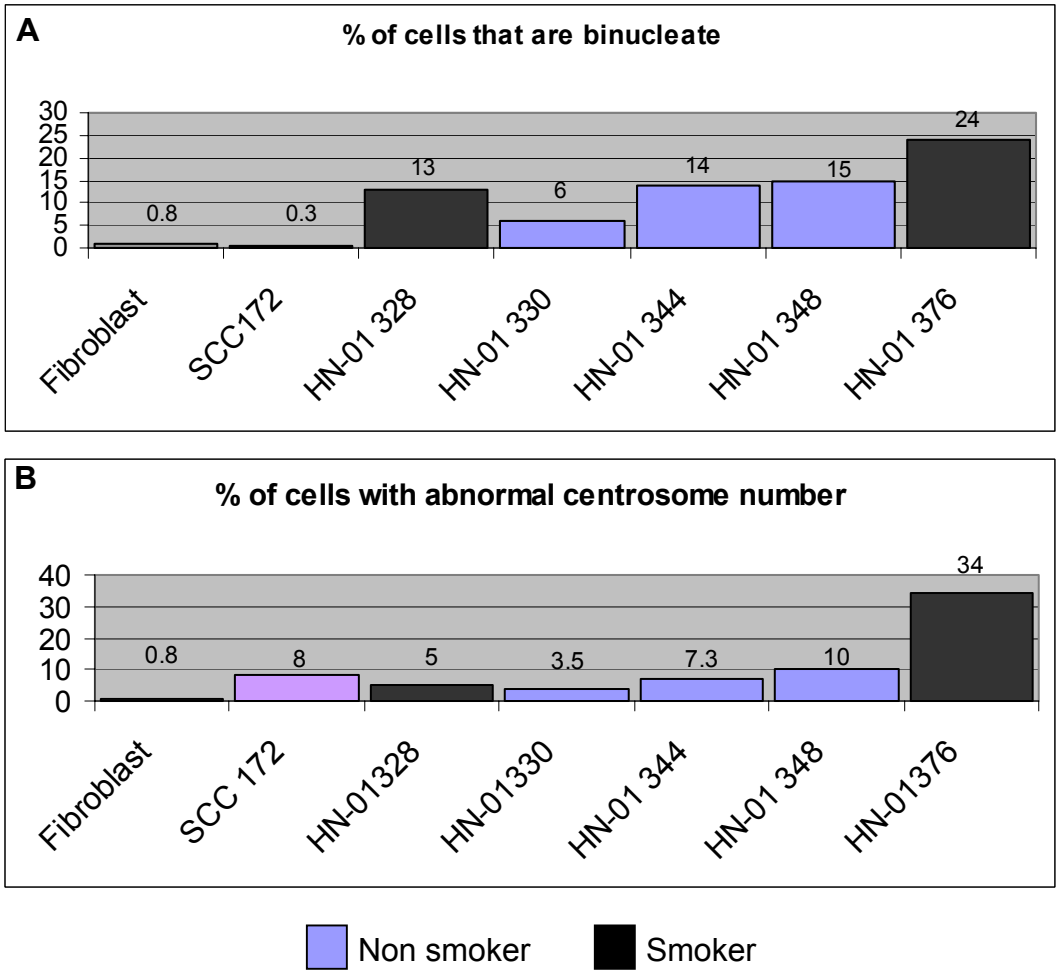


Figure 15: Centrosomal amplification in oral keratinocytes.

A: the frequency of binucleate cells observed in 1000 interphase cells.

B: the frequency of cells containing greater than two γ-tubulin signals (a centrosomal marker for) observed in 500 interphase cells.



3.2 NuMA localization and expression

Next, we examined NuMA localization and found that NuMA was seen throughout the interphase nucleus including micronuclei (figure 16A,B) as previously described in other cancer cells (Compton, Szilak et al. 1992). NuMA protein was excluded from nucleoli in interphase cells. In mitotic cells, NuMA signal was found at all spindle poles during metaphase and anaphase including all the poles of multipolar cells (figure 13D). Little difference could be seen in the overall expression levels by IMF but some variations of NuMA localization were observed in the different cell lines. For example, some UPCI:SCC78 cells contained NuMA aggregates or a complete bar of NuMA across the metaphase plate (figure 16D,E).

Figure 16: Localization of NuMA.

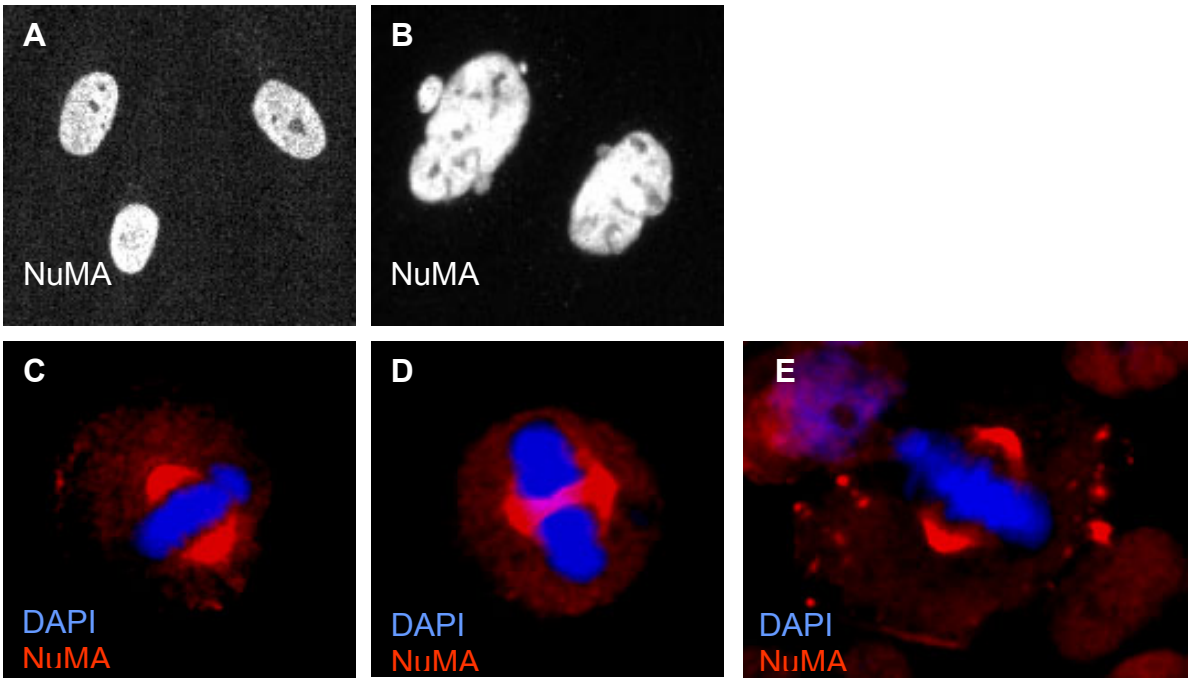
A: interphase fibroblasts labeled with anti-NuMA antibody,

B: NuMA stained interphase oral cancer cells,

C: a normal appearing metaphase oral cancer cell (UPCI:SCC70),

D: a metaphase UPCI:SCC78 with a 'bar' of NuMA,

E: a metaphase UPCI:SCC78 with NuMA aggregates.

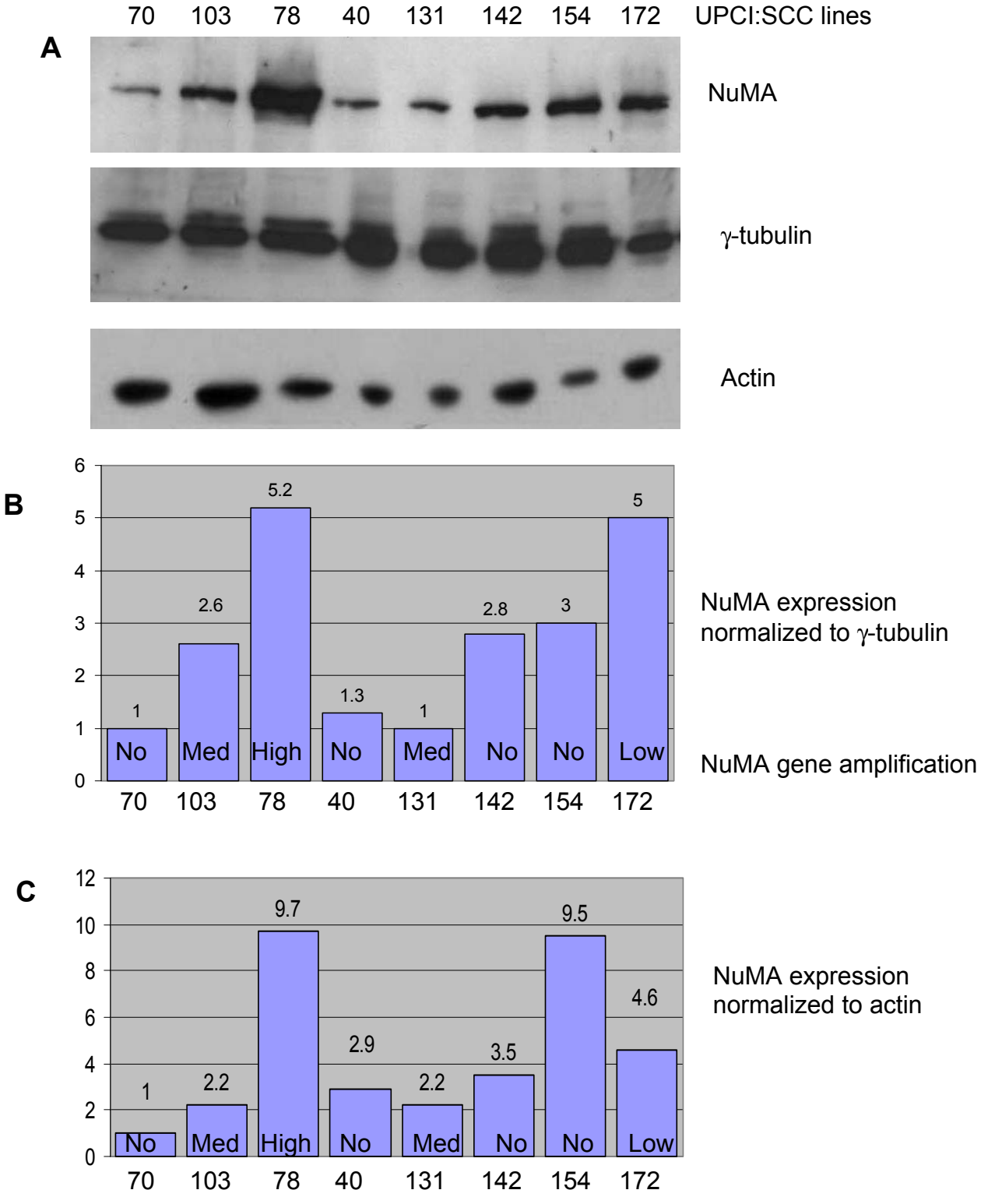


We then used immunoblot analysis in order to compare NuMA protein expression levels between the different oral cancer cell lines. The pericentriolar protein, γ-tubulin, was chosen to compare the level of NuMA expression to another centrosomal protein. We also compared NuMA expression to actin expression as a means of determining NuMA levels versus total cellular protein. SCC78 and SCC172 had consistently high levels of NuMA expression when compared to either total protein or another centrosomal protein (figure 17A). SCC70, SCC40 and SCC131 had the lowest levels of NuMA expression when compared to γ-tubulin. The remaining lines had intermediate levels of expression. Differences were seen in expression levels depending on which loading control was used for comparison. This may be explained by some cell lines having higher levels of NuMA at each centrosome. Alternatively, some cell lines may express less actin, in particular, SCC78 and SCC154. These two cells lines do not adhere as monolayers and form non-motile colonies unlike any of the other six cell lines. Some transformed cells are known to have a reduction in the expression of actin microfilaments (Darnell et al., 1995). SCC78, the line with the highest NuMA expression, also contained NuMA aggregates and unusual localizations patterns when examined with IMF suggesting that excess NuMA may aggregate and/or affect the mitotic spindle. In general, it appeared that NuMA expression varied between the cell lines independently of another centrosomal protein. Thus, it is possible that NuMA may be involved in the observed variations in the frequencies of segregational defects.

Since NuMA gene amplification had already been observed in these oral cancer cell lines by our collaborators (Xin Huang and Susanne Gollin, unpublished results), we attempted to correlate gene amplification to protein expression. The quantification of expression levels is shown in figure 17B with the gene amplification listed for each line. Gene amplification did not appear to correlate to protein expression in most cell lines. Although, SCC78 had high gene amplification and high protein expression, most of the other lines did not show any correlations. When comparing to actin expression, there also did not appear to be a correlation between gene amplification and protein expression per cell (figure 17C). NuMA expression levels do seem to vary between cell lines independently of another centrosomal protein, γ-tubulin, but these changes do not seem to be correlated to amplification level.

Figure 17: Comparison of NuMA expression and gene amplification.

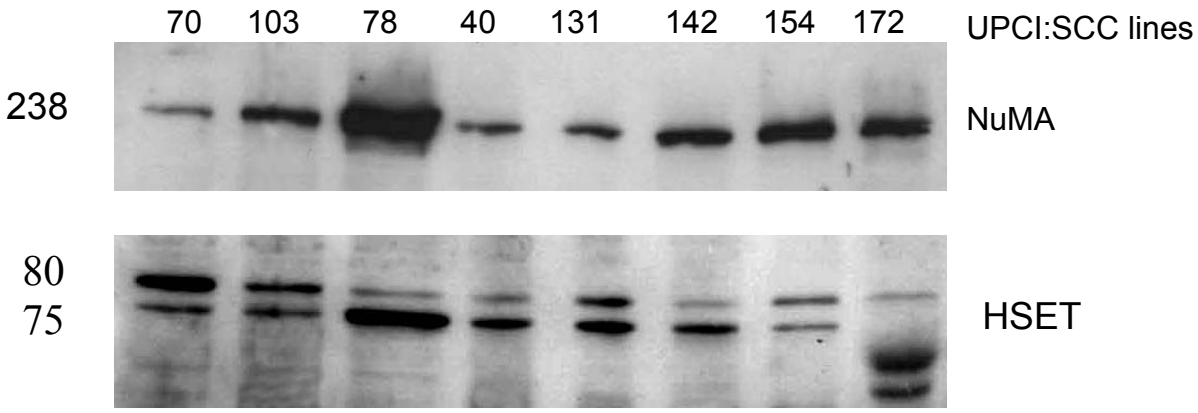
A: Typical western blot of UPCI:SCC lines using anti-NuMA, anti-γ-tubulin, and anti-actin, **B**: Quantification of NuMA expression normalized to γ-tubulin with gene amplification level shown below. **C**: Quantification of NuMA expression normalized to actin.



3.3 Expression of HSET

I then attempted to determine whether NuMA is over-expressed exclusively by comparing to another related centrosomal protein. HSET is involved in spindle pole organization. It is a minus-end directed kinesin-like motor protein that has been shown to have some overlapping functions with NuMA in chromosome movement (Gordon, Howard et al. 2001). By indirect immunofluoresence, HSET had very similar localization patterns in oral cancer cells as in normal keratinocytes (data not shown). When examined by western blotting, HSET appears to have variable expression patterns between the different oral cancer lines (figure18). Previously it has been shown in HeLa cells that the HSET antibody recognizes two proteins with equal intensity at 75 and 80 kDa (Mountain, Simerly et al. 1999). Interestingly, the OSCC cells do not appear to have similar expression patterns. Two OSCC lines, 103 and 131, appear to have equimolar expression but the majority of OSCC lines consistently expressed one of the two forms of HSET at lower levels. One line, 172, appears to have higher expression of the 75kDa form with degradation unlike any of the other lines. These two HSET proteins have shown to be two different isoforms with identical sequence except for the fourteen C-terminal amino acid residues (Mountain, Simerly et al. 1999). It is known that HSET is a member of a kinesin-related protein family that have C-terminal motor domains. As the two isoforms of HSET differ only in their putative motor domains they may have different activities and expression differences may be significant in spindle formation or maintenance. It has been shown that the 75 kDa isoform of HSET, purified from HeLa cells, is an active microtubule motor that can slowly move microtubules *in vitro* (DeLuca, Newton et al. 2001). The 80 kDa isoform has not been characterized. At present it is not known how the two isoforms are produced or whether they differ in function. Although HSET appears to have less variability in total expression then NuMA between the different cell lines, the differences in isoform expression may be significant.

Figure 18: Comparison of NuMA and HSET expression;
Western blot using anti-NuMA and anti-HSET antibodies

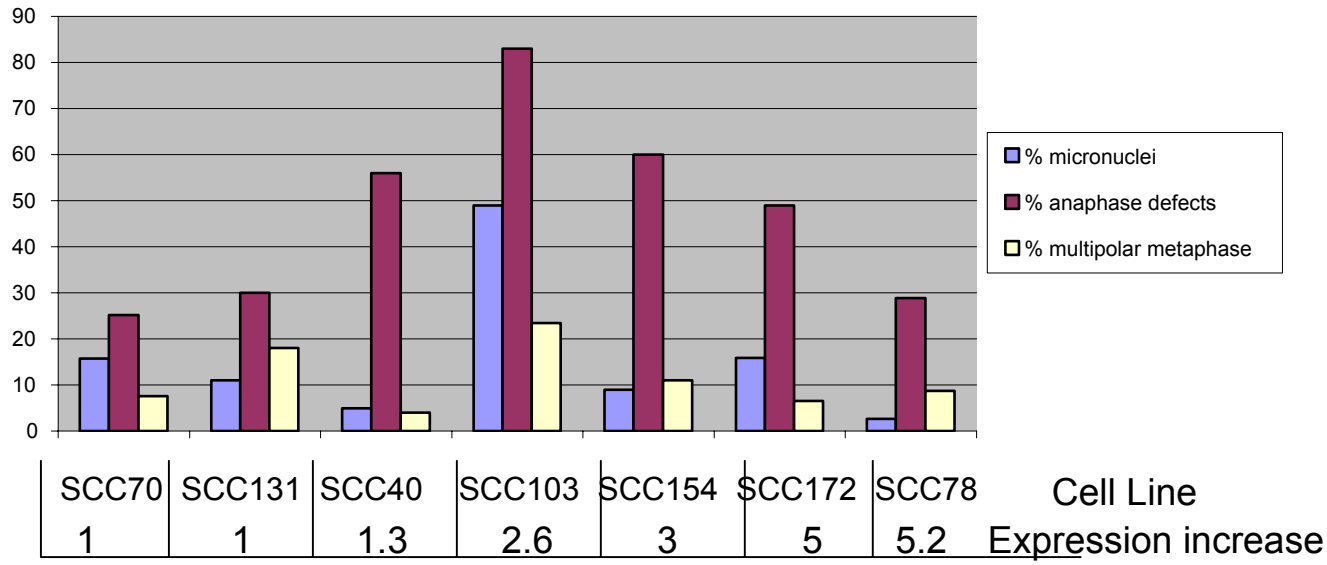


3.4 NuMA expression and spindle defects

We then examined whether this variable protein expression of NuMA correlates to changes in the frequencies of spindle defects. The frequencies of multipolar spindles, anaphase defects (including bridges and lagging chromosomes) and micronuclei in each cell line are shown in figure 19. The cell lines are listed in increasing order of NuMA expression. There did not appear to be any direct correlation between NuMA expression and the frequencies of multipolar spindles. The frequency of anaphase defects did rise with increasing expression until a maximum was reached with UPCI:SCC103, a cell line with an intermediate elevation of expression.

Figure 19: Segregation defects and NuMA expression.

The average frequencies of micronuclei, anaphase defects and multipolar spindles is shown for each oral cancer cell line. 1000 interphase cells were examined for micronuclei. 100 metaphases and 50 anaphases were examined for defects. The NuMA expression level per centrosome and normalized to UPCI:SCC70 (the cell line with the lowest NuMA expression) is shown beneath each cell line.



4. Discussion:

I observed centrosomal defects in both primary epithelial cells and cancer cells. Higher frequencies of excess centrosomes and binucleate cells were seen in a few primary cultures derived from smokers. It is possible that these defects may be an early change in cells resulting from the damaging effects of smoking however the mechanism of smoking induced changes is unclear. Higher variability in cell karyotypes was seen in normal oral keratinocytes in another example of possible smoking induced damage (Barrera, Ai et al. 1998). Centrosome amplification, binucleation, and karyotype changes may be early markers for the damage caused by smoking before any dysplastic cell formations can be seen. It is possible that binucleate cells may be an early step in increasing ploidy, and then subsequent aneuploidy and karyotype changes. This increase in ploidy may cause a destabilization of chromosomal segregation by

making it more likely for mitotic defects to occur. Or alternatively, segregation defects are more likely to be tolerated in cells with increased chromosome numbers. For example, if a diploid cell undergoes mitosis with segregation defects and chromosome loss is the result, that cell may be less likely to tolerate this loss than if the same event happened to a tetraploid cell. An aneuploid cell also has altered ratios of gene expression which may be a key factor in producing cells with a survival advantage. If cells are able to divide with the chromosomal and centrosomal amplification we observed in binucleate cells, then segregation defects and the karyotypic changes may be the result.

My data did not provide evidence of NuMA expression being directly correlated to its gene amplification. One explanation may be that the use of quantitative PCR for determining amplification and the use of Western blotting for determining expression are only representing average values. A single cell with very high levels of NuMA expression may increase the overall average of expression but may only be seen as a single segregation defect. Higher amplification leading to higher expression and subsequent defects may be occurring in a subpopulation of cells. Possible support of this is that FISH analysis of individual cells showed a subpopulation of cells with much higher than average NuMA amplification (Xin Huang and Susanne Gollin, unpublished results). Another explanation for the lack of correlation may be that NuMA is not involved in segregation defects. Alternatively, other mechanisms may be involved and interact with NuMA to produce defects but the complicated interactions may mask any direct correlations. Finally, NuMA may be activated by post-translational modification or interaction with other proteins obscuring a correlation to protein levels.

When examining whether NuMA expression has an effect on spindle defects I observed an increase in anaphase defects until an intermediate expression level was reached. This may be evidence that excess NuMA is involved in causing defects until a saturation point where the cell either can not divide or excess NuMA aggregates and ceases to function. NuMA aggregates were observed in cell lines with very high expression. Although NuMA expression does not appear to correlate to gene amplification or spindle defects directly, it does appear to vary between cell lines independently of other centrosomal proteins. Over-expression of NuMA may have a role in spindle defects but it is probably not the only mechanism involved.

Chapter VI. SUMMARY AND CONCLUSIONS:

We have developed an effective system for analyzing mitotic segregation defects in living cells. This labeling and imaging system has allowed us to observe cells dividing with segregation defects and determine the fate of these cells. We found that cells dividing with defects are viable. Mitotic defects are not simply artifacts of dying cells. Anaphase bridges do not halt the separation of segregating chromosomes, micronuclei do not appear to interfere with division, and cells are able to divide with multipolar spindles, giving these cells the potential to contribute to chromosomal instability. Anaphase bridges break early in anaphase and lead to the formation of micronuclei. For these chromosomal fragments to form micronuclei, the anaphase bridge must have broken at more than one site. This breakage and removal of DNA fragments from the main nucleus results in chromosomal instability. Micronuclei are transcriptionally inactive and thus gene expression from these DNA fragments is lost also resulting in chromosomal instability. We observed a cell forming a multipolar spindle divide with clearly uneven segregation to the daughter cells. This evidence of chromosome breakage, dicentric chromosomes, micronuclei formation and loss of gene expression suggest that chromosomal instability caused by segregational defects is an ongoing process in these cancer cells.

Mitotic delays were observed in cells dividing with defects. While the mitotic spindle checkpoint appeared to be active, it did not appear to be functioning properly as some cells were able to enter anaphase without correcting their defects. Anaphase bridges activated a mechanism that produced a metaphase delay. This mechanism of delay may be a novel checkpoint or a previously uncharacterized aspect of the known spindle checkpoint. Our data suggests that these cells have maintained at least a partial function of the spindle checkpoint, but this incomplete checkpoint allows cells to proceed with defects resulting in chromosomal instability.

While NuMA protein expression varied between cell lines we did not find any correlations between gene amplification and protein expression. We also were unable to provide evidence of any correlations between variations in NuMA protein expression and variations in the frequencies of segregation defects.

In summary, we have shown that cells dividing with these mitotic segregation defects produce viable cells and these defects contribute to chromosomal instability.

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