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IN VITRO ANALYSIS OF CULTURED BARRETT'S ESOPHAGUS CELLS: INSIGHTS INTO MECHANISMS OF GENOMIC INSTABILITY AND POSSIBLE THERAPEUTIC STRATEGIES

Maria Corinna Palanca-Wessels

A dissertation submitted in partial fulfillment
of the requirements for the degree of

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Abstract

IN VITRO ANALYSIS OF CULTURED BARRETT'S ESOPHAGUS CELLS: INSIGHTS INTO MECHANISMS OF GENOMIC INSTABILITY AND POSSIBLE THERAPEUTIC STRATEGIES

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Barrett's esophagus is a human premalignant condition in which the normal squamous epithelial lining of the esophagus is replaced by a metaplastic columnar epithelium. Patients with Barrett's esophagus are at increased risk for the subsequent development of esophageal cancer. While some of the genetic and cell cycle abnormalities that occur in progression to cancer in Barrett's esophagus have been delineated through the analysis of patient tissue samples, there has been no relevant *in vitro* or animal model that has been developed in which to investigate the mechanisms contributing to carcinogenesis in this condition. We describe the development of a cell culture protocol that allows the establishment of Barrett's esophagus and normal squamous esophagus epithelial cultures from endoscopic biopsies. Through these methods, we have been able to establish the first long-term Barrett's epithelial cultures that contain the same genetic and cell cycle abnormalities present *in vivo*, including the inactivation of the p53 and p16 tumor suppressor genes and the elevation of the 4N DNA content fraction. We used four of the Barrett's cell strains to study the contribution of centrosome abnormalities, tetraploidization, and telomerase activation to neoplastic progression in Barrett's esophagus, focusing on the effect that these factors may have on genomic instability. The establishment of Barrett's cell strains provides an *in vitro* model in which to test promising therapeutics in the treatment or prevention of esophageal cancer. We performed a series of experiments testing the efficacy of chemotherapeutic drugs and a mutant E1B adenovirus in selectively killing Barrett's cells lacking p53 function. The results show the feasibility of using the Barrett's cell strains for *in vitro* therapeutic screening purposes.

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I would like to thank all my colleagues who have helped me with my graduate project, particularly past and present members of the Peter Rabinovitch and Brian Reid labs. In particular, I would like to thank Carissa Sanchez, Michael Barrett and Patty Galipeau from the Reid lab and Katherine Gollahon and Martin Poot from the Rabinovitch lab. I have also been fortunate to have worked with many undergraduate students who have contributed to various portions of my project: Katie Rohrer Carter, Charlotte Hubbert, Christine Kim, Aga Luczak, Eric Muller, Christina Jahnke and Patrick Solari. In addition, I owe a debt of gratitude to my family (including wonderful in-laws) who have helped me in a multitude of ways in this educational journey. Finally, I would like to thank my husband Sean who has supported and encouraged me along the way and has helped to make this accomplishment possible.

Dedicated to

my parents Benruben and Helena

and my loving husband Sean

Introduction

Barrett's esophagus is a condition in which the normal squamous epithelial lining of the esophagus is replaced by a metaplastic columnar epithelium. Patients who are diagnosed with Barrett's esophagus are at increased risk for progression to cancer. This progression is characterized by an accumulation of cell cycle and genetic changes that can be followed over time by periodic endoscopic surveillance which involves the procurement of biopsies that are used for clinical diagnosis as well as research study. Because of this, Barrett's esophagus has proven to be an excellent model for the study of human neoplastic progression *in vivo*. In order to extend this work, we have established Barrett's esophagus cell strains for *in vitro* studies that will provide a system that is amenable to testing hypotheses derived from *in vivo* observations. This may lead to better therapeutics for the treatment and/or prevention of cancer in this condition.

Patients diagnosed with Barrett's esophagus generally present with symptoms consistent with gastroesophageal reflux disease (GERD). Irritation of the esophageal lining due to recurrent reflux of the acidic contents of the stomach leads to frequent episodes of heartburn. Inadequacy of the lower esophageal sphincter located at the juncture of the esophagus and stomach, commonly exacerbated by the presence of a hiatal hernia, is often the cause. Diagnosis of Barrett's esophagus is based on an endoscopic visual examination in which the highly vascularized pink mucosa that typifies Barrett's columnar epithelium can be seen to invade the pale white mucosa typical of normal stratified squamous epithelium. The diagnosis is further confirmed by histologic examination that shows columnar epithelium in esophageal biopsies. The cell type of origin in Barrett's esophagus is still unknown. Cytokeratin studies have suggested that Barrett's epithelium may originate from a multipotential stem cell that has the ability to differentiate into either a squamous or columnar type epithelium (Boch, 1997; Salo, 1996). These studies showed that Barrett's epithelium expresses cytokeratin 13, a marker of squamous differentiation, in addition to cytokeratins 8 and 18, which are markers of columnar epithelium. It is thought that the columnar epithelium, which resembles the cell type lining the rest of the gastrointestinal tract, is more resistant to acid exposure than the normal

squamous lining of the esophagus. Patients are often prescribed medications and lifestyle changes aimed at reducing the exposure of the esophagus to acid in order to ameliorate their symptoms. In some cases, Barrett's epithelium regression can be observed, but the appearance of squamous epithelial islands within a Barrett's segment does not necessarily correlate with a better prognosis.

The primary concern for both patient and clinician is the elevated risk of adenocarcinoma in Barrett's esophagus. Whereas squamous cell carcinoma of the esophagus is typical of smoking-associated neoplasia, esophageal adenocarcinoma almost entirely presents itself in the setting of Barrett's esophagus changes. The enhanced risk of esophageal adenocarcinoma is between 30 to 40 fold compared to the general population (Spechler, 1986). The past two decades have shown a steady increase in the incidence rate of esophageal adenocarcinoma (Hesketh, 1989; Blot, 1991, 1993). Surgical resection of the esophagus is warranted once an adenocarcinoma has been detected. Barrett's associated esophageal cancer has a poor prognosis, with a five-year survival typically 7% (Miller, 1973-89), unless an early superficial cancer is diagnosed by endoscopic surveillance, in which case five-year survival rates of 80% to over 90% can be achieved after surgical resection (Rusch, 1994; Paraf, 1995; Levine, 1993; Clark, 1996). Even so, the timing of esophagectomy remains controversial (Rusch, 1994; Rice, 1993). Some clinicians advocate the removal of the esophagus in patients diagnosed with high grade dysplasia, whereas others prefer to wait for the detection of early curable cancer since not all patients with high grade dysplasia progress to cancer and because esophagectomy is associated with substantial morbidity and mortality. Preoperative multimodality therapy consisting of two cycles of 5-fluorouracil and cisplatin in addition to radiation may improve survival, but results from randomized studies do not show a clear benefit (Walsh, 1996; Bolton, 1998). Similarly, experimental techniques for the endoscopic ablation of the Barrett's epithelium, including multipolar electrocoagulation (MPEC) and photodynamic therapy, have not been found to reduce cancer risk consistently (van den Boogert, 1999). Periodic endoscopic surveillance of Barrett's esophagus patients is recommended since this improves the probability of the detection of early intramucosal cancer. The frequency of endoscopy is dependent on the degree of abnormality present in a particular patient. Biopsies obtained during endoscopy enable histologic and flow cytometric analyses within

the Barrett's segment. One protocol which has been successful in detecting small cancers, thus dramatically improving patient survival, consists of dividing each level of the esophagus into four quadrants and obtaining biopsies from each of the quadrants at two centimeter intervals (Levine, 1993). This minimizes the chance of a small cancer being missed and dramatically increases patient survival following surgical resection of the esophagus. A similar protocol has been used in the study of esophagectomy specimens in which the resected esophagus is laid flat and tissue samples removed in a grid-like pattern. This has allowed a detailed analysis of the cell cycle and molecular changes that occur over time in a patient, as well as the diversity of changes present within a single esophagectomy specimen (Rabinovitch, 1988; Reid, 1992; Blount, 1994; Barrett, 1999).

Data obtained from endoscopic biopsies and surgical resection specimens have delineated a sequence of changes that are associated with neoplastic progression in Barrett's esophagus (Barrett, 1999). Generally, an elevation of cycling cells evidenced by an elevation in Ki-67 positive cells is observed early in the course of the disease (Reid, 1993). Subsequently, increased flow cytometric 4N (G2/tetraploid) fractions are detected (defined as >6% of the total cell population based on receiver-operator curves). 17p and 9p loss of heterozygosity frequently occurs in conjunction with inactivation of the tumor suppressor gene p53 (typically by mutation) and the cyclin-dependent kinase inhibitor p16 (by either mutation or promoter hypermethylation) (Blount, 1994; Neshat, 1994b; Barrett, 1996b; Wong, 1998). Late changes include the development of aneuploidy and 5q loss of heterozygosity (Blount, 1993). Other loci found to be frequently lost include 18q and 13q (Barrett, 1996a). The relevant genes on 5q, 18q, and 13q that contribute to progression have not been found. These affected gene products are important since they may represent additional therapeutic targets in the treatment or prevention of esophageal adenocarcinoma.

Until therapies are developed to forestall the progression towards cancer in Barrett's esophagus, endoscopic surveillance remains the centerpoint of disease management. Adequate surveillance of patients at higher risk requires frequent endoscopy with large numbers of biopsies examined. Histologic grading of dysplasia is currently used in order to attempt to stratify patients according to

cancer risk and focus the greatest medical resources on the subset of patients in greatest need. There is, however, need for additional objective markers of cancer risk, such as might be provided by flow cytometry or molecular genetic analyses. Recently, long-term follow-up study has shown that DNA content abnormalities (aneuploidy) and elevated G2/tetraploid fractions can provide additional predictive assessment of risk in Barrett's esophagus (Reid et al, submitted). Elevated G2/tetraploid fractions are particularly robust predictors, and there is some evidence that they may be associated with p53 loss of heterozygosity and mutation (Galipeau, 1996).

Lacking an animal model of Barrett's esophagus, studies using short-term *ex vivo* biopsy or cell culture studies have been performed (e.g. Fitzgerald, 1996; Garewal, 1990). Establishment of long-term Barrett's esophagus cultures has been difficult and only a handful of reports describing culture of Barrett's have been described (Garewal, 1992a; Washington, 1994; Wang, 1996; Khan, 1997). The use of *in vitro* cultures to investigate possible mechanisms contributing to progression is highly desirable but up till now has been impossible due to the inability to maintain the cells in culture for an adequate period of time.

The goal of my project was to develop a method to establish Barrett's epithelial cultures in order to provide an *in vitro* system for the study of Barrett's esophagus. Chapter 1 describes the protocol used to establish five Barrett's cell strains; four of which were subsequently genetically characterized and used for the remainder of the project. Additional cell strains were also established by using refinements to the protocol. The development of culture methods to grow esophageal squamous cells and esophageal fibroblasts that may be used as control cells in future experiments is also included in Chapter 1. In Chapter 2, the initial cell cycle and genetic characterization of the Barrett's cell strains are described. The majority of the data in Chapters 1 and 2 is work that was previously published (Palanca-Wessels, 1998). The Barrett's cell strains can be used to investigate possible mechanisms contributing to progression to cancer. This was the focus of my work described in Chapter 3 in which I examined the possible roles of tetraploidization and centrosome abnormalities in neoplastic progression in Barrett's esophagus. In Chapter 4, I describe the transduction of the Barrett's cell

strains with a retroviral construct containing the human telomerase reverse transcriptase (hTERT) gene. These studies were intended to examine the effect of activating telomerase in a premalignant cell strain containing early genetic abnormalities and investigate the contribution of telomerase in neoplastic evolution. The final chapter contains preliminary data on possible therapeutics designed to target molecular abnormalities found in Barrett's esophagus. This work investigated the feasibility of using the cell strains as an *in vitro* system for screening therapeutic agents prior to clinical trials.

In summary, by designing a protocol for the establishment of Barrett's esophagus cell strains, new avenues of research into the mechanism and prevention of neoplastic progression in Barrett's esophagus have been opened. It is my hope that future studies will utilize the cell strains that have been (and will be) established and that this will lead to improvements in the prevention and treatment of esophageal cancer.

Chapter 1: In Vitro Culture of Barrett's Epithelial Cells

Introduction

Barrett's esophagus has several features that have made it a suitable model for the study of neoplastic progression *in vivo*. First, the tissue of interest is accessible and biopsies can be removed for further histologic, flow cytometric and genetic study. Second, patients return periodically for endoscopic surveillance allowing one to monitor changes in the nature of the tissue over time. Third, once a cancer has been detected, surgical removal of the esophagus is performed. The esophagectomy specimen can be laid out and tissue samples removed and analyzed, thus allowing a mapping of the entire esophagus which contains the spectrum of neoplastic changes, from metaplastic to dysplastic to malignant. By comparing the flow cytometric and genetic differences between a single patient's samples over time or within an esophagectomy specimen, an ordering of the events which occur in cancer progression in Barrett's esophagus has been developed (Reid, 1992; Reid, 1993; Barrett, 1999; Jankowski, 1999 review).

While the data generated from the analysis of patient endoscopic biopsies has been substantial and has led to hypotheses regarding possible mechanisms that could be involved in neoplastic evolution in Barrett's esophagus, this research method has limitations. Epithelial cell number and purity in endoscopic biopsies are concerns, although there are ways to minimize these problems. More importantly, biopsies represent snapshots in time, and while the data is suggestive of a plausible sequence of events, the causal interaction between these changes remains speculative. In order to test a possible mechanism, one would like to be able to have a system that is able to be easily manipulated and studied. Lacking a good animal model, an *in vitro* cell culture system is the only feasible alternative in the case of Barrett's esophagus.

For this reason, we attempted to develop cell strains derived from endoscopic biopsies of Barrett's esophagus patients. At the start of this project, there were only two reports from groups claiming to have been able to maintain short-term cultures of Barrett's esophagus cells (Garewal, 1992a; Washington, 1994). There has been subsequently two other reports describing growth of Barrett's

esophagus *in vitro* (Wang, 1996; Khan, 1997). Short-term Barrett's epithelial cultures have been used for karyotypic analyses, determination of growth factor receptor expression, and evaluation of drug effects (Garewal, 1989; Garewal, 1990; Garewal, 1992b). None of these cultures, however, have been shown to retain the molecular genetic abnormalities that can lead to cancer in Barrett's esophagus *in vivo*. Additionally, some of the experiments that would be desirable to perform require extended culture *in vitro* and large cell numbers which are impossible to obtain with short-term cultures.

We have isolated Barrett's epithelial cells from endoscopic biopsies obtained from patients with Barrett's esophagus and have successfully grown these cells in continuous culture for several months. After establishing the initial Barrett's esophagus long-term cultures, we attempted to optimize our tissue processing technique and culture conditions in order to improve the success rate of cell strain establishment (11% success rate). In order to compare the phenotypic differences between cultured Barrett's esophagus at different stages of neoplastic progression, it will be necessary to establish cell strains representing earlier as well as later stages of progression. We manipulated the culture medium and tried different methods to improve primary culture growth. We found that processing the biopsy as soon as possible after procurement was important and that fibroblast feeder layers may improve success rates. We also found that use of esophageal fibroblast conditioned media allowed us to reduce the concentration of bovine pituitary extract in the media. By making changes in the protocol, the success rate for establishment of cultures was increased from 11% to 40%, and four additional Barrett's esophagus cell strains were obtained for future analyses. We determined that tissue procured from esophagectomy specimens was not ideal for the establishment of cultures. Instead, endoscopic biopsies processed as soon as possible after procurement yielded the best results.

In addition to establishing Barrett's esophagus cell cultures, we wanted to develop control cell strains to represent the normal *in vivo* condition, either from the non-Barrett's segment of Barrett's esophagus patients or from individuals without Barrett's esophagus. However, a difficulty in Barrett's esophagus is that the normal cell type in the esophagus is stratified squamous epithelium

which is radically different from the columnar metaplastic epithelium of Barrett's esophagus. The identity of the progenitor Barrett's cell is unknown, although immunohistochemical studies using cytokeratin markers suggest that either squmous or columnar cells may arise from a multipotential stem cell present in the basal layer of the normal esophageal squamous epithelium (Boch, 1997; Salo, 1996). There is no "normal" Barrett's esophagus type cell in the human esophagus. The esophageal squamous cell does represent the normal cell type lining the esophagus and represents a clinically relevant control cell in terms of therapeutics. Certain experiments, however, can not be performed due to the inherent differences present between a columnar versus a squamous cell type. For example, using gene expression arrays to search for candidate genes that contribute to carcinogenesis would be difficult since the expression pattern of columnar cells are much different from the squamous cells in ways that are not due to differences in cancer susceptibility. The other possible control cell type is the esophageal fibroblast. An advantage in using the fibroblast is that a large amount of research has been perfomed using normal human fibroblasts and much is known about its *in vitro* biology. In addition, the conditioning of Barrett's esophagus cell culture media by esophageal fibroblasts, as mentioned above, allows the reduction of bovine pituitary extract concentration. It is therefore desirable to be able to maintain a reliable supply of fibroblasts for this purpose. Most importantly, both esophageal squamous epithelial cells and fibroblasts can be isolated from endoscopic biopsies, and thus can be the source of isogenic controls for Barrett's cell strains. We were able to develop a protocol that allows us to reliably isolate and grow short-term cultures of esophageal squamous cells, as well as to grow long-term cultures of esophageal fibroblasts for use as control cells and for the conditioning of Barrett's esophagus cell culture media.

Results

Primary culture of Barrett's epithelium

Attempts were made to initiate forty-four primary cultures from thirty-nine different patients. With five patients, more than one attempt was made to establish primary cultures using biopsies obtained on different endoscopy dates. The culture medium used for initiation of the primary cultures was a modification of a medium described previously (Washington, 1994). The medium consisted of

MCDB 153 modified by the addition of fetal bovine serum, hydrocortisone, epidermal growth factor, cholera toxin, bovine pituitary extract, L-glutamine, insulin-transferrin-selenium and antibiotics (see Chapter 1 Methods). Most cultures were able to be maintained for one week and showed a significant number of cells explanting from tissue fragments (Figure 1.1A). After one week, unsuccessful cultures generally showed degeneration of epithelial cells, characterized by vacuolation and cytoplasmic enlargement, accompanied by fibroblast overgrowth. No molecular or cytogenetic characterization was performed on unsuccessful cultures due to limited cell number and fibroblast overgrowth. Doubling time of short-term cultures was unable to be calculated since these cultures did not reach second passage. Four cultures of Barrett's epithelium (CP-52731, CP-94251, CP-18821, and KR-42421) survived long-term passaging. Three of the four cultures (CP-52731, CP-18821, CP-94251) were initiated from biopsies obtained from a region of high-grade dysplasia while biopsies for the remaining culture (KR-42421) were obtained from a region of non-dysplastic metaplasia. Phase microscopy of cells in a representative long-term culture at confluency is shown in Figure 1.1B.

Patient records were reviewed to determine whether there were characteristics that distinguished the patients whose cells were successfully cultured from those whose cells did not survive *in vitro*. There was no correlation between the degree of histologic abnormality within the Barrett's segment and the ability to culture the biopsies from that particular region in the patient's esophagus. However, 17p LOH *in vivo* was statistically associated with the ability to establish long-term culture. Twenty of 23 unsuccessful cultures were from patients without 17p LOH, whereas 3 out of 4 successful cultures were from patients having 17p LOH ($p=0.025$; Fisher's exact test).

The cultures were continuously passaged and showed an initial period of steady proliferation before reaching a plateau phase of growth after 17 to 29 population doubling levels (CP-94251, PDL 29; CP-52731, PDL 28; KR-42421, PDL 24; CP-18821, PDL 17), indicative of replicative senescence. Doubling time of the cultures at early passages ranged from 2 to 4 days extending to 7 to 14 days at very late passages. A culture was considered senescent if confluency was not reached after one month of undisturbed growth in the same tissue culture flask with regular re-feeding. The long-term cultures

at this stage showed increased debris in the growth medium, and the cells acquired altered morphology, with cytoplasmic enlargement or elongation. Senescent cultures containing viable cells have been maintained for up to four months without giving rise to immortal clones.

Confluent cultures stained with hematoxylin and eosin (H&E) possessed an epithelial-type morphology. H&E stained slides of the long-term cultured Barrett's esophagus cells at late passage were examined by a pathologist experienced in reviewing histologic sections of Barrett's esophagus and esophageal adenocarcinoma (Dr. Rodger C. Haggitt, University of Washington). There was no evidence for dysplastic change based on cellular morphology in any of the four Barrett's esophagus cell cultures. Positive immunocytochemical staining for cytokeratin was established by flow cytometry utilizing a FITC-conjugated antibody to cytokeratins 8 and 18, which are expressed at high levels in columnar epithelium but minimally in stratified squamous epithelium. Expression of cytokeratins 8 and 18 has been shown in Barrett's and gastric epithelium but not in the normal squamous epithelium of the esophagus (Boch, 1997; Salo, 1996). Cytokeratin-positive epithelial cells comprised at least 88% of cells in all four established cultures. Although it is uncertain whether the cells negative for cytokeratin 8 and 18 are non-epithelial, this is consistent with the visual presence of a minor fibroblast component at early passage. None of the Barrett's cell cultures examined expressed mucin that could be detected with Alcian blue staining. However, it has been shown previously that dysplastic Barrett's epithelium *in vivo* can have defects in mucin production at the histologic and ultrastructural level (Levine, 1989).

Improvements on Barrett's esophagus primary culture technique

Although five Barrett's esophagus cell strains were established initially, the success rate for establishment of long-term cultures was only 11%. Furthermore, the cell strains exhibited characteristics of the same stage of neoplastic progression i.e. after p53 and p16 inactivation (see Chapter 2). To obtain a more representative collection of Barrett's esophagus cell strains, in collaboration with Dr. Marilyn Cornwell and Christina Jahnke, we attempted to make improvements

on our published primary culture technique that would allow us to more reliably establish cultures from various stages of neoplastic progression.

Primary culture establishment can be divided into a series of steps which includes disaggregation of the tissue, plating of cells with subsequent cell adherence, and cell growth initiation and maintenance in a formulated medium. Our original published protocol consisted of digesting endoscopic biopsies in collagenase I for five hours, followed by gentle trituration and rinsing of the digested tissue, then plating of the resulting cell and tissue suspension in uncoated tissue culture flasks with modified MCDB media (Palanca-Wessels, 1998). A number of variations on primary culture establishment were tried in order to optimize these steps. The protocol variations for a series of ten patient samples and the results from each are summarized in Table 1.1 .

Different disaggregation methods were utilized including collagenase II at shorter incubation in case the long incubation in collagenase III reduced cell viability. With this treatment, however, few cells adhered and these were eventually lost with passaging. With esophagectomy specimens, we tried mincing the tissue rather than using enzymatic treatment. Since the tissue from a surgical sample was already friable and largely necrotic, we reasoned that prolonged exposure to enzymatic digestion might negatively affect cell viability. Again, few tissue pieces adhered to the plate and fibroblastoid cells were the only cells that grew out. None of the changes described above improved the cell growth from the original protocol.

Another variable that could affect primary culture establishment is the ability of the cells to attach to the tissue culture flask. We had tried human fibronectin coated plates in our initial attempts at primary culture establishment, but had found no difference in cell culture establishment compared to cells seeded on uncoated plastic (Palanca-Wessels, 1998), although we have found that coating glass slides with human fibronectin enhanced cell attachment to this particular surface. In another attempt to increase cell attachment, plates were coated with gelatin and cell attachment to these plates and

uncoated plates was compared. There appeared to be no difference in cell adherence between the plates.

We also varied the protocol method in order to improve growth of the Barrett's epithelial cells while minimizing fibroblast growth. We tried lowering the concentration of fetal bovine serum in the media since fibroblasts require serum for optimal proliferation. We reduced the serum concentration from 5% to either 2.5% or 1%, but these cultures were lost to contamination so we were unable to determine whether this was successful in inhibiting fibroblast growth. In another approach, we tried combinations of KBM (a serum-free medium containing growth factors that is used for esophageal squamous cells) and MCDB media in order to lower the serum concentration while providing additional growth factors. In the patient samples where this protocol was tried, epithelial cell growth was only seen in the wells containing a 1:4 ratio of KBM to MCDB but not in the 1:1 ratio. There was, however, no cell growth in the regular MCDB formulation to use for comparison. The cells from this protocol looked more compact and less granular compared to other protocols. Taking a different strategy, we also tried conditioning the Barrett's MCDB media with an established Barrett's cell strain reasoning that there may be autocrine factor(s) released by Barrett's epithelial cells that might aid epithelial cell proliferation while inhibiting fibroblast growth, particularly since Barrett's epithelial cells appear to grow best at a high cell density. Media was conditioned for 24 hours and centrifuged to remove floating cells before adding an equal volume of fresh MCDB culture media. This 1:1 mixture was used to feed primary Barrett's cultures. No difference in the growth of primary cultures fed either Barrett's epithelial cell conditioned media or regular media was observed.

Another approach we tried was the use of feeder layers. Irradiated esophageal fibroblast feeder layers were used in order to provide a cell matrix for adhesion and as a source of possible soluble factors that might aid cell proliferation. Use of fibroblast feeder layers might also prevent or minimize the attachment of fibroblasts from the biopsies. Esophageal fibroblasts were irradiated with 20 Grays and plated at 100%, 75%, and 50% confluency one day prior to procurement of biopsies. The wells containing feeder layers at 50% confluency appeared to have the most cells adhered to the plate.

HS27a cells, a bone stromal cell line known to secrete transforming growth factor-β, was also used as a feeder layer in the same protocol as the esophageal fibroblasts and appeared to give encouraging results similar to those found when using esophageal fibroblasts. Esophageal fibroblasts, however, are the normal cells adjoining Barrett's epithelium *in vivo* and may, therefore, be more appropriate for use as a feeder layer than bone stromal cells.

We were able to establish four more Barrett's esophagus cultures out of ten attempts for an improved success rate of 40% compared to 11% previously. These were designated CJ-3635, CJ-7818, CJ-8477, and CJ-9358. The initial genetic characterization of the four "new" cultures (performed in collaboration with Laura Prevo) suggests that the new culture variations may be more permissable for growth of cells lacking p53 and/or p16 abnormalities compared to the original protocol. We flow sorted cells from each of the cultures into 2N and 4N samples, then performed 9p and 17p LOH analyses as well as p53 gene sequencing of exons 5 through 9. Only one (CJ-9358) of the four cultures contained 17p LOH and none of the cultures contained 9p LOH. No mutations in exons 5 through 9 in the p53 gene were found in any of the cultures. One of the cultures (CJ-3635) did contain a base change in the intronic sequence past exon 9 of the p53 gene, but this most likely is a germ-line polymorphism. At the same time, we performed genetic analysis on the last Barrett's cell strain (CP-4213)that was established using the original protocol but which was not characterized with the four other strains (CP-94251; CP-52731; CP-18821; KR-42421) also established by the same method (see Chapter 2). Like the majority of the cell strains established by this method, we found that CP-4213 contained a p53 gene mutation (Leu→His; codon 130); this is the same p53 mutation found in the patient's *in vivo* biopsies.

Improvements on Barrett's esophagus cell culture maintentance

Since esophageal fibroblast feeder layers enhanced Barrett's esophagus primary culture growth, this suggested that esophageal fibroblasts secreted growth factors that stimulated the proliferation of Barrett's esophagus cells. Since previous clonogenic experiments using the regular MCDB growth media had been unsuccessful, we wanted to test whether using media conditioned by other cell types

(especially esophageal fibroblasts) would help clone formation. We tried transwell plates which consist of a membrane-lined insert that is placed in a regular six-well plate. The feeder cells were plated in the bottom of the wells whereas the Barrett's cells were plated within the insert. The pores in the insert were too small for Barrett's cells to cross, but allowed soluble factors secreted by the feeder cells into the media to diffuse across the two compartments. HS5 bone stromal cells, esophageal fibroblasts, Hela cervical cancer cells, telomerase-expressing Barrett's cells (see Chapter 4) and parental non-telomerase expressing Barrett's cells were all plated separately, each in one well of a six-well transwell plate. Barrett's esophagus cells were plated at low density within the inserts and examined under a light microscope for growth. After one month, there was still no growth in any of the wells. Thus, co-culturing of the cells did not appear to increase colony forming efficiency.

Because transwells are expensive and allow limited cell growth, the use of conditioned media rather than co-culture of cells to enhance cell growth appeared to be a more feasible alternative. Although co-culture of cells did not enhance the clonogenicity of Barrett's epithelial cells, we tried to determine whether conditioned media would improve the growth of established Barrett's cultures, particularly since the established cell strains have relatively slow doubling times (48 to 72 hours). Furthermore, conditioning the media could possibly allow the reduction of some of the costly growth factors in the media such as bovine pituitary extract (see below). We compared the growth of Barrett's cells fed media conditioned by different cell types. Media was harvested after 24 hour incubation in flasks containing HS27a bone stromal cells, normal human esophageal fibroblasts, HeLa cervical cancer cells, SW480 colon cancer cells, and Barrett's esophagus cell strains. The media was centrifuged to remove floating cells and an equal volume of fresh MCDB media added prior to feeding the Barrett's esophagus cultures. Triplicate cultures were grown for one week before being harvested and cells counted. Results are shown in Figure 1.2. Compared to control cultures, esophageal fibroblast conditioned media conferred a two-fold increase in Barrett's cell culture growth compared to control. Barrett's culture conditioned media gave a one and a half fold increase in cell growth. Media conditioned by HeLa, SW480 and HS27a did not improve growth compared to control. Morphologically, the cells grown in esophageal fibroblast, Barrett's epithelial cell and HS27a bone

stromal cell conditioned media appeared smaller and less granular than cells in the control wells, a morphology that is conventionally associated with vigorous growth and "healthy" culture.

In order to confirm the results from the conditioned media experiments and determine whether there was any additive benefit in using both fibroblast and Barrett's conditioned media, we performed another growth experiment. We used fibroblast and Barrett's epithelial cell conditioned media for 24, 48, or 72 hours individually, or combined in a 1:1 ratio with each other. Additionally, we used media that had been mixed with KBM esophageal squamous media at 1:4 and 1:1 ratio with regular MCDB media because this had appeared to help primary cell culture establishment as described previously. Cells were grown in triplicate for five days while being fed with the different formulations of media and then harvested and counted. Results are shown in Figure 1.3. Growth in fibroblast conditioned media was enhanced by longer conditioning times, whereas growth in Barrett's cell conditioned media decreased with longer conditioning relative to control. The esophageal fibroblast 24 hour conditioned media results in this experiment were less than what was seen in the previous experiment in Figure 1.2 (200% versus 100% of control growth). This was most likely due to the lower confluency (50% versus 100%) of the esophageal fibroblast culture that was used for conditioning the media in this experiment. Presumably, a higher confluency in the culture used for conditioning media would have increased the amount of growth factors secreted and would have resulted in a similar enhancement of growth as seen previously. Growth with a mixture of both fibroblast and Barrett's cell 48 hour conditioned media was slightly higher than control. Growth in KBM containing media was the same as control.

Because esophageal fibroblast conditioned media improved Barrett's epithelial cell growth, we wondered whether conditioned media would allow the reduction of growth factor supplements without a negative effect on Barrett's cell growth. Bovine pituitary extract (because of its expense) was an attractive supplement to test. CP-94251 Barrett's esophagus cells were plated and grown in either MCDB growth media containing the normal concentration of bovine pituitary extract (BPE) or in a mixture of half fibroblast conditioned MCDB media containing no BPE and half fresh MCDB

media containing the normal concentration of BPE (for a final BPE concentration of 50% of normal). Cells were harvested and counted at various timepoints within a 24 day period. The growth curves of cells grown in both media formulations are shown in Figure 1.4. Conditioning of media allowed the reduction in the amount of BPE in the growth medium with minimal effect on the proliferation of the Barrett's epithelial cells. Reduction of BPE to 50% of the normal concentration in unconditioned media inhibited CP-94251 cell growth by 40% relative to cells fed with unconditioned media containing the full amount of BPE (see Chapter 4, Figure 3.4).

Culture of control cells: esophageal squamous epithelial cells and fibroblasts

In order to establish esophageal squamous cultures, we used a variation of a method used to culture oral squamous tissue by Oda et al (Oda, 1998). We obtained esophageal squamous tissue biopsies from Barrett's esophagus patients at the time of endoscopy and processed these as soon as possible after procurement. Tissue was digested overnight in dispase and forcefully pipetted in order to disaggregate the tissue and obtain a primarily single cell suspension. The cells were then plated in KBM media with BEGM SingleQuots™ (a serum-free media containing growth factors) and allowed to attach. The majority of cells did not adhere to the tissue culture flask. These were primarily differentiated squames which could be identified under the light microscope as large, flat cells with small nuclei. The few cells that did attach were most likely the stem cells that comprised the basal layer of the stratified epithelium. These cells were much smaller in size than the differentiated squames and were more spherical and refractile under phase microscopy. After about a week in culture, small islands of epithelial cells began to proliferate. These were the squamous esophageal cells (Figure 1.5A). We found that it was best not to allow the cells to become confluent since this induced differentiation and cessation of growth. The esophageal squamous cultures could be passaged up to five times. Although we have been able to reproducibly culture normal esophageal squamous cells, the *in vitro* lifespan of these cells has been short, precluding experiments that require extended cell culture or vast numbers of cells.

Growth of esophageal fibroblasts from endoscopic biopsies has not been difficult. From the initial attempts of establishing primary cultures from Barrett's esophagus tissue biopsies, fibroblast growth has been evident in early passages. To grow fibroblasts, the normal protocol for establishing Barrett's esophagus cultures was followed. Instead of using MCDB growth media, DMEM containing 10% fetal bovine serum was used. Fibroblast growth was evident within a few days of culture (see Figure 1.5B). These cells could be passaged and fed as usual. For conditioning media, fibroblasts could be maintained in MCDB growth media lacking bovine pituitary extract for extended periods of time without detriment.

Discussion

In this chapter, we have described the development of techniques for the establishment and maintenance of premalignant Barrett's epithelial cell strains. These cell strains represent the only premalignant Barrett's epithelial cell strains that have been described as being able to be maintained in long-term culture. We have examined possible methods in which to improve the growth of Barrett's cells *in vitro* including the use of feeder layers and conditioned media. By using variations on the original protocol we have improved the success rate for establishment from 11% to 40% and have obtained cultures that do not have p53 or p16 abnormalities. We also described the culture of esophageal squamous epithelial cells and fibroblasts from endoscopic biopsies for use as isogenic controls (and for the possible development of organotypic cultures—see Chapter 5 discussion). The Barrett's cell strains are an opportunity to investigate areas that have been impossible to study without animal models or a manipulable *in vitro* system.

While we have refined the original protocol for initiation of primary cultures, further improvements will be necessary to ultimately establish cell strains representing the spectrum of neoplastic progression in Barrett's esophagus. As a first step, we have established four cell strains using the improved to protocol that do not contain p53 and p16 abnormalities and thus represent an earlier stage in Barrett's esophagus than the cell strains established using the original protocol. There appears, however, to be additional growth factors that are lacking in the culture media. These may be

paracrine factors secreted by the underlying esophageal stromal cells, since conditioning of the media by fibroblasts appears to benefit cell growth and allows the reduction of bovine pituitary extract in the media. These growth factors may prevent the cessation of cell growth (and possibly terminal differentiation) of the Barrett's esophagus cells that explant from the biopsy fragments. Ideally, isogenic cell strains established from several regions of a single esophagectomy specimen (representing various stages of neoplastic progression and clonal lineages) would provide an excellent *in vitro* model in which to examine mechanisms contributing to genetic instability in Barrett's esophagus. At the present time, however, this remains unattainable since epithelial cell viability is low in esophagectomy specimens, perhaps due to the time lag between resection and the removal of tissue samples for processing. We found that endoscopic biopsies processed shortly after removal yielded the best results. An intriguing possibility, mentioned in Chapter 4, is the use of retroviral transduction of the catalytic subunit of human telomerase in the establishment of Barrett's esophagus cells and/or control cells. Extending the *in vitro* lifespan and enhancing the proliferation of Barrett's epithelial cells by telomerase activation may allow the design of experiments which require large numbers of cells and provides a way ensuring that the cultured cells from a particular patient will not be depleted.

Normal human control cells are important in any experiment in which one wants to compare the characteristics or behavior of an abnormal cell type. For Barrett's esophagus, two cell types seem most appropriate, both related anatomically to the cell of interest: the squamous epithelial cell and the fibroblast. The squamous epithelial cell is an ideal "clinically relevant" control if one wants to compare, for example, the effect of an experimental therapeutic, since it is the normal cell type lining the esophagus. However, present culture techniques allow only limited passage of esophageal squamous cells before terminal differentiation occurs. This prevents the use of these cells in experiments that require large cell numbers or extended culture. The esophageal fibroblast is an attractive control cell since fibroblasts are used widely in the scientific community and much more is known about the *in vitro* behavior of this cell type than the squamous epithelial cell. Furthermore, fibroblasts can undergo more population doublings than squamous cells *in vitro*, making it possible to

use these cells for experiments requiring large cell numbers. The main drawback in using fibroblasts is that epithelial and fibroblast cells have been shown to behave in different ways and it is wrong to assume that conclusions drawn from one cell type are applicable to the other. Fortunately, we have found that both esophageal squamous cells and fibroblasts can be cultured easily from biopsies obtained during endoscopy using cell culture techniques that we have optimized. Thus, we have the potential ability to culture normal esophageal squamous cells and fibroblasts as well as Barrett's esophagus cells from the same patient. In fact, we have been able to do this with tissue from at least one of our most recent patients. The ability to obtain normal control cells from the same patient allows the design of experiments where it is desirable to have isogenic cells for comparison. In conclusion, we have demonstrated the feasibility of establishing both Barrett's cell cultures and appropriate control cell cultures from endoscopic biopsies. In subsequent chapters, we show the utility of the Barrett's cultures as an *in vitro* system for the study of mechanisms contributing to neoplastic progression (Chapters 3 and 4) and the investigation of promising therapeutics (Chapter 5).

Methodology

Biopsy specimens

Tissue samples were obtained during routine endoscopy of patients with Barrett's esophagus. These patients were evaluated as part of a prospective endoscopic cancer surveillance study approved by the Human Subjects Division of the University of Washington in 1982 and renewed annually thereafter. A total of six to twelve biopsies from each patient were taken within the Barrett's segment for culture. Additional biopsies from both the Barrett's segment and normal gastric mucosa were obtained for histology, flow cytometric and molecular analyses. Specimens for tissue culture were immediately placed in Minimal Essential Medium (Sigma, St. Louis, MO) on ice prior to processing.

Initiation of Barrett's esophagus primary cultures

Biopsies were processed within two hours of procurement. Two methods for initiation of cultures were used, which are modifications of techniques described previously (Washington, 1994; Garewal, 1992)). Both methods (explant and enzymatic) were used successfully in the establishment of

primary cultures from one patient (52731) while the enzymatic method was used for the other patient biopsies (94251, 18821, 42421).

In the explant method, biopsies were minced into fragments of approximately 1 to 2 mm^3 in size. The pieces of tissue were placed in a 100 mm tissue culture dish and anchored by a sterile glass microscope slide before addition of growth medium (see below). In the enzymatic method, whole biopsies were placed in a T-25 flask containing 1 mg/ml collagenase III (Worthington, Freehold, NJ) in 1X phosphate-buffered saline and incubated at 37°C with gentle agitation for one hour then syringed in order to dissociate the tissue. The resulting suspension was plated onto uncoated plastic tissue culture dishes or dishes coated with human fibronectin (Sigma). No difference in the attachment or growth of cells on uncoated versus fibronectin-coated plates dishes was observed.

A modification of the enzymatic method was used to establish primary cultures from the three other patients (94251, 18821, 42421). Biopsies were minced into pieces approximately 1 mm^3 in size and placed in T-25 flasks containing 1 mg/ml collagenase III in modified MCDB 153 (see below) and incubated at 37°C for 5 hours. After gentle trituration and rinsing, the resulting suspension was transferred to uncoated T-25 flasks.

Culture medium and maintenance of Barrett's esophagus cells

The culture medium used for initiation and maintenance of primary cultures has been described previously (Washington, 1994). MCDB 153 (Sigma) was modified by the addition of 5% fetal bovine serum, 0.4 µg/ml hydrocortisone (Sigma), 20 ng/ml epidermal growth factor (Gibco, Grand Island, NY), 10^{-10} M cholera toxin (Sigma), 140 µg/ml bovine pituitary extract (Sigma), 100 units/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), and 0.25 µg/ml amphotericin B (Gibco). Other modifications to the media not previously described include: 4 mM L-glutamine (Gibco), 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium (Sigma). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C and fed three times a week.

Initial trypsinization occured at least three weeks after initiation in the enzymatic method and after six weeks in the explant method. Differential trypsinization was done in order to control fibroblast overgrowth in primary cultures and was performed as follows. After incubation in trypsin for three minutes, floating cells were removed from the tissue culture flask and fresh trypsin added. After incubation for an additional three minutes, detached cells were removed, additional trypsin added, and the remaining attached cells incubated for a final three minutes. The cells harvested from each individual round of trypsinization were kept separate and returned to culture. Cultures derived from the last round of trypsinization generally contained a higher proportion of epithelial cells compared to earlier rounds of trypsinization.

Cultures initiated by the methods described above have been kept separate since the initial isolation of cells and are designated CP-52731, CP-94251, CP-18821, and KR-42421. Cultures were split no greater than 1:4 after growth to over 90% confluency with an average split time of one month. Cells from all cultures have survived more than twenty passages and have been maintained continuously in culture between twelve to seventeen months.

Histochemistry and cytokeratin staining

Cells were stained with H&E and Alcian blue using standard histochemical techniques. Cytokeratin staining was performed using a flow cytometric method. 5×10^5 cells were fixed with 1% EM grade methanol-free formalin for ten minutes on ice. Cells were rinsed with PBA (phosphate-buffered saline containing 0.1% bovine serum albumin fraction V) and then treated with 0.1% Triton-X in PBA and incubated for three minutes on ice. After rinsing cells with PBA, fluorescein isothiocyanate (FITC)-conjugated anti-cytokeratin antibody CAM 5.2 (Becton Dickinson, San Jose, CA) or FITC-conjugated isotype-matched mouse immunoglobulin (FITC IgG2a; Becton Dickinson) for negative controls was added and left to incubate on ice for forty-five minutes in the dark. After incubation, cells were rinsed with 0.1% Triton-X in PBA and then resuspended in 10 μg/ml 2,4 diamidino-6-

phenylindole (DAPI; Accurate Chemical and Scientific Corp., Westbury, NY) before analysis. For each of the Barrett's cell lines, the level of FITC fluorescence in cells stained with FITC IgG2a, representing non-specific background antibody staining, was used as the threshold for cytokeratin positivity for that particular cell line.

Primary culture of squamous epithelial cells or fibroblasts from the esophagus

For squamous cell cultures, at least six biopsies were obtained from the non-Barrett's esophagus region of the patient's esophagus during endoscopy. Biopsies were immediately placed in cold MEM after procurement. The tissue was then placed in supplemented KBM media containing 4 mg/ml dispase II (Boehringer Mannheim, Indianapolis, IN) overnight in a 37°C incubator as previously described for oral epithelium (Oda, 1998). Supplemented KBM media consists of KBM media without calcium (Clonetics, San Diego, CA) containing one vial of BEGM SingleQuots™ (Clonetics) that includes bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, transferrin, insulin, triiodothyronine, and GA-1000. Retinoic acid included in the BEGM SingleQuots is not added to the media. In addition, L-glutamine (20 mM) and calcium chloride (0.05 mM) is added to the media. After incubation, the biopsies were removed and placed in fresh media and triturated until the tissue fragments could pass through the tip of a ten milliliter pipet and the suspension contained mainly single cells. The cells and fragments were pelleted and suspended in supplemented KBM growth media and placed in tissue culture flasks and returned to 37°C incubator containing 5% CO_2. Cells were fed three times weekly and passaged before reaching confluency.

Esophageal fibroblasts were established using the same protocol as Barrett's esophagus cultures except growth media was DMEM containing 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. Cells were grown in a 37°C incubator containing 5% CO_2. Cells were fed three times weekly.

Use of irradiated feeder layers and transwell plates

For irradiated feeder layers, feeder cells (either HS27a bone stromal cells or normal esophageal fibroblasts) were irradiated with 20 Grays then plated and allowed to adhere overnight in six-well tissue culture plates. Processed cells and tissue pieces from endoscopic biopsies were plated on the feeder cells and fed three times weekly.

For co-culture experiments, 2×10^5 conditioning cells were plated separately in each well of a 24 mm transwell plate (Corning Costar Corporation, Cambridge, MA) and allowed to adhere and grow until confluent. Conditioning cells used were HS27a, HeLa, SW480, and established Barrett's cell strains were used. 9×10^3 Barrett's esophagus cells were plated within the transwell insert and fed with Barrett's growth media three times weekly.

Preparation of conditioned media and measurement of cell growth

Media was conditioned by incubating media with confluent or near-confluent cultures for 24 to 72 hours. The media was harvested by removing it from the cultures and centrifuging it at 2000 rpm for 15 minutes in order to remove any floating cells. The media was transferred to a new flask (leaving behind about 2 mls of media and any pelleted cells), then supplemented with an equal volume of unconditioned fresh modified MCDB media. Additional fetal bovine serum (2.5 mls per 100 mls media) was added to the 1:1 mixture of conditioned and fresh MCDB media in order to replenish serum growth factors that may have been depleted by the cells during conditioning. At various times after plating in conditioned media, Barrett's cells were trypsinized and harvested in minimal volumes then counted visually using a hemocytometer. Experiments were performed in duplicate or triplicate.

Table 1.1 Variations on the published Barrett's esophagus primary culture protocol and culture success

	Tissue source	Disaggregation method	Growth surface	Culture media	Culture success
1	Biopsy	• Collagenase II • Collagenase III	• Gelatin coated plastic • Uncoated plastic	• Conditioned MCDB • MCDB media	(-)
2	Biopsy	• Collagenase III	• Esophageal fibroblast feeder layer • Uncoated plastic	• MCDB media	(+)
3	Biopsy	• Collagenase III	• Uncoated plastic	• MCDB/KBM • MCDB media	(+)
4	Biopsy	• Collagenase III	• Uncoated plastic	• MCDB reduced serum • MCDB media	(-)
5	Esophagectomy	• Mincing • Collagenase III	• Uncoated plastic	• MCDB media	(-)
6	Biopsy	• Collagenase III	• HS27a feeder layer • Uncoated plastic	• MCDB media	(+)
7	Biopsy	• Collagenase III	• HS27a feeder layer • Uncoated plastic	• MCDB media	(+)
8	Biopsy	• Collagenase III	• Uncoated plastic	• MCDB media	(-)
9	Biopsy	• Collagenase III	• Esophageal fibroblast feeder layer • Uncoated plastic	• MCDB media	(-)
10	Esophagectomy	• Collagenase III	• Esophageal fibroblast feeder layer • Uncoated plastic	• MCDB media	(-)

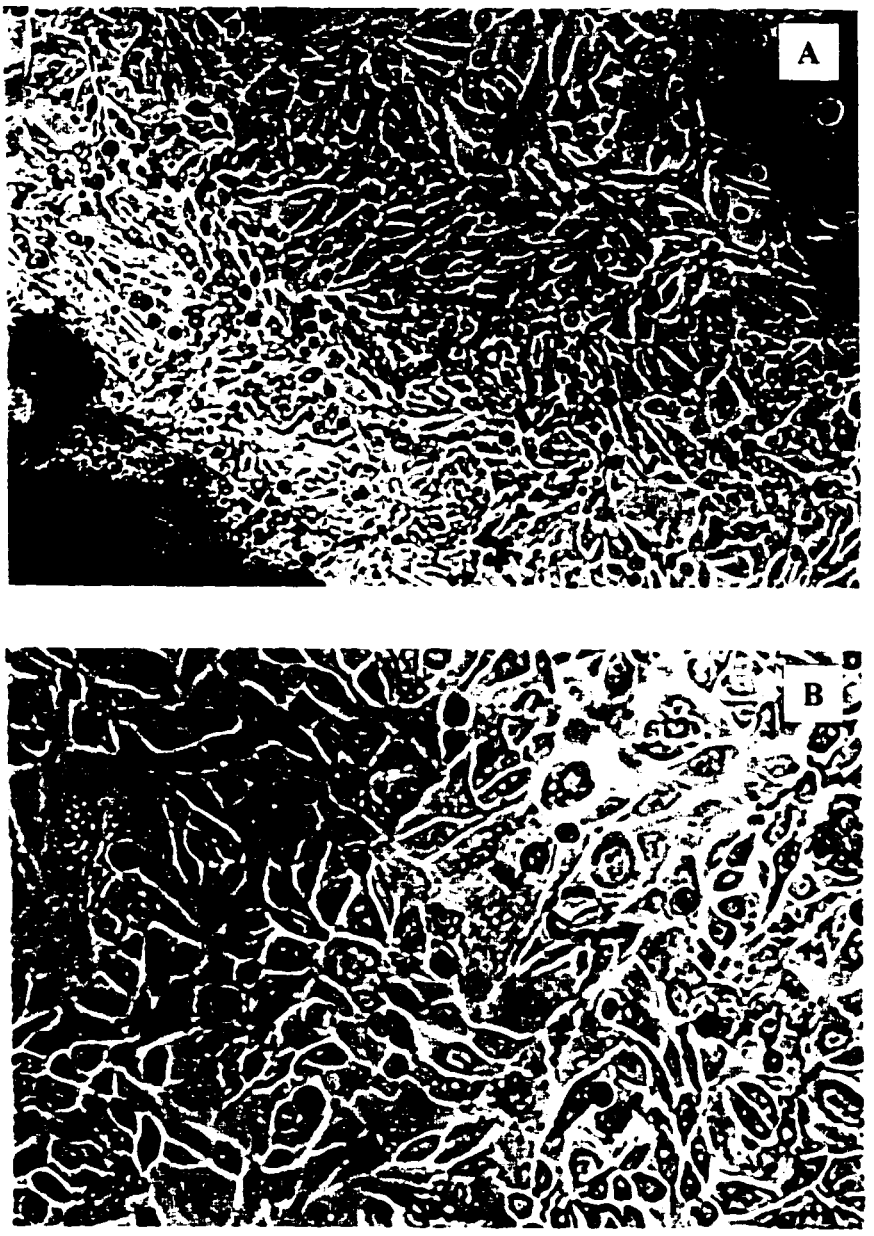


Figure 1.1 Phase contrast microscopy of Barrett's epithelial cell cultures. The monolayer cultures show a cobblesone-like morphology at confluency which is typical of epithelial cells. *A*, a primary culture showing cells explanting from pieces of tissue after 7 days in culture, this particular culture was eventually overgrown by fibroblasts subsequent to the first passage; *B*, a representative long-term Barrett's epithelial culture, CP-94251, at passage 8.

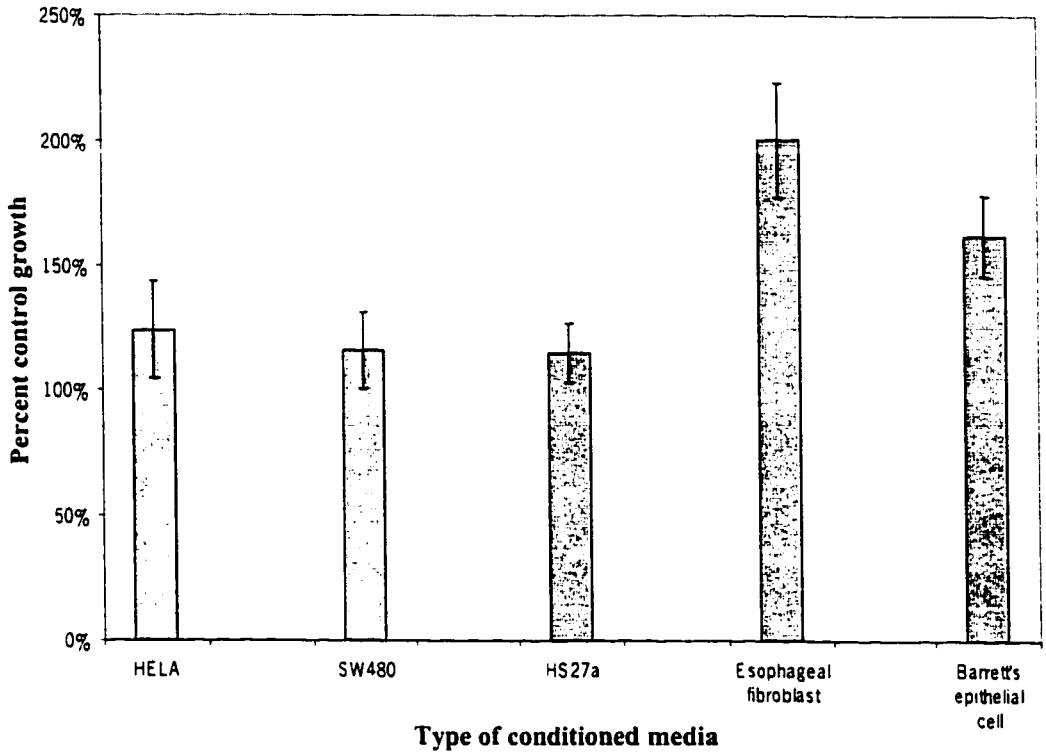


Figure 1.2 Comparison of Barrett's epithelial cell strain growth in media conditioned by various cell types. MCDB media was conditioned for 24 hours by HeLa, SW-480, HS27a, esophageal fibroblast, or Barrett's epithelial cells, then diluted 1:1 with fresh media. Barrett's esophagus cells were grown in the 1:1 mixture of fresh and conditioned media for one week prior to harvest. The percent of cell growth relative to cells grown in unconditioned media is shown. Y-error bars represent the standard deviation of triplicate samples.

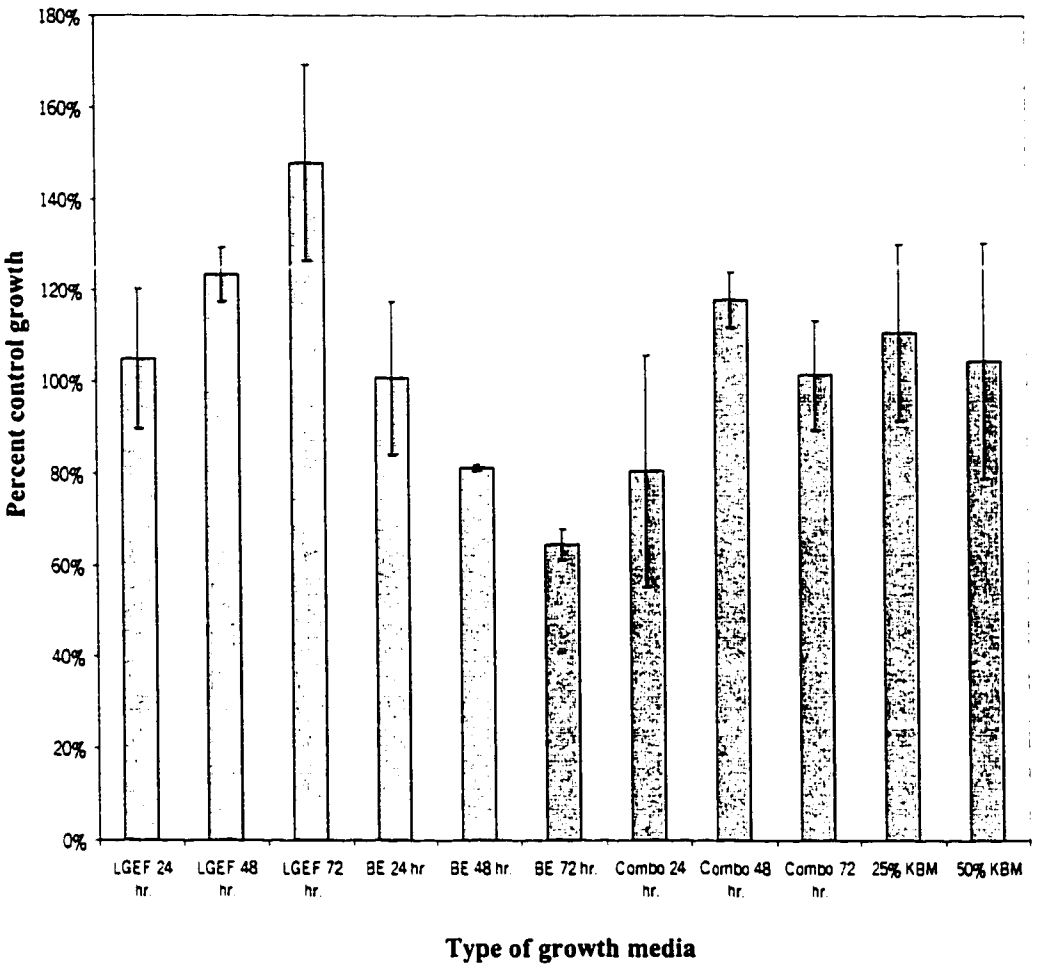


Figure 1.3 Comparison of Barrett's epithelial cell growth in various formulations of conditioned MCDB media or unconditioned KBM/MCDB combined media. For conditioned media, MCDB was incubated with esophageal fibroblasts (LGEF) or Barrett's esophagus cells (BE). Conditioned MCDB media was then combined with an equal volume of fresh MCDB media. For wells fed with combined media (Combo), equal volumes of LGEF and BE conditioned media were mixed prior to addition of fresh MCDB media. For KBM/MCDB media, KBM was mixed with unconditioned MCDB to either 25% or 50% of the final volume. Cells were counted after five days of growth in the various media formulations. Values represent the percent of control (unconditioned MCDB) cell growth. Y-error bars represent the standard deviation of triplicate samples.

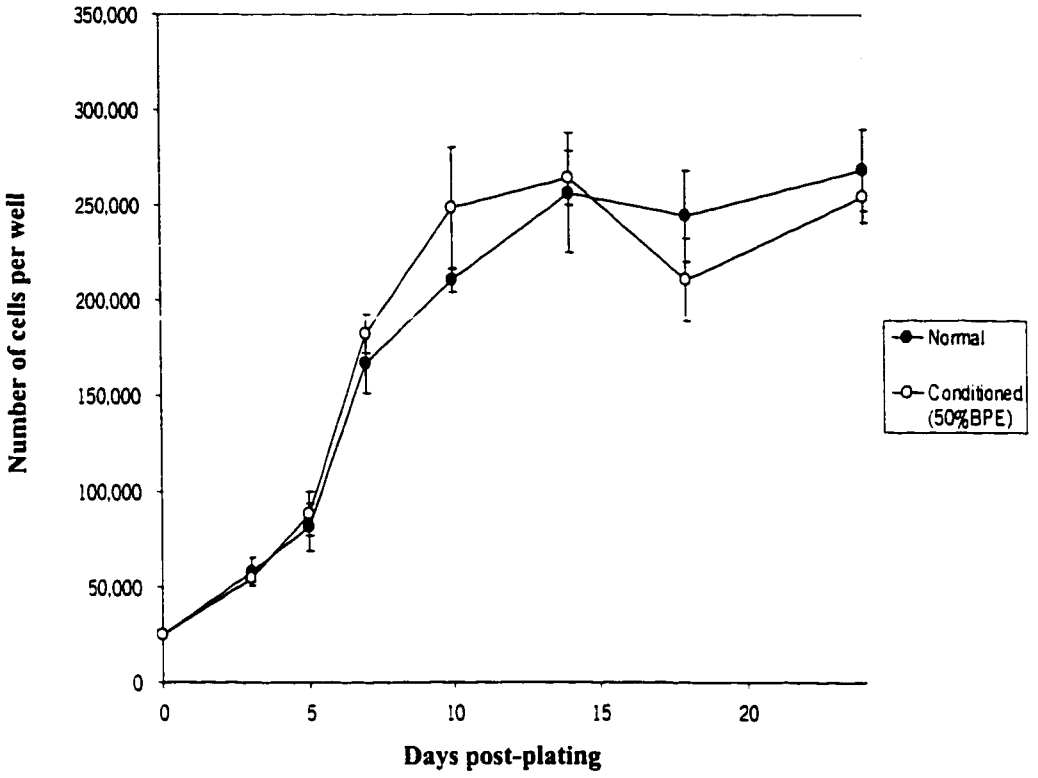


Figure 1.4 Growth curves of Barrett's epithelial cells grown in normal growth media or conditioned media containing 50% of the normal concentration of bovine pituitary extract (BPE). Cells were plated and fed with either regular MCDB containing the normal concentration of BPE or a 1:1 mixture of fibroblast conditioned to fresh MCDB media containing 50% of the normal BPE concentration. Cells were harvested over a 24 day period and the total number of cells per well recorded. Y-error bars represent the standard deviation of triplicate samples.

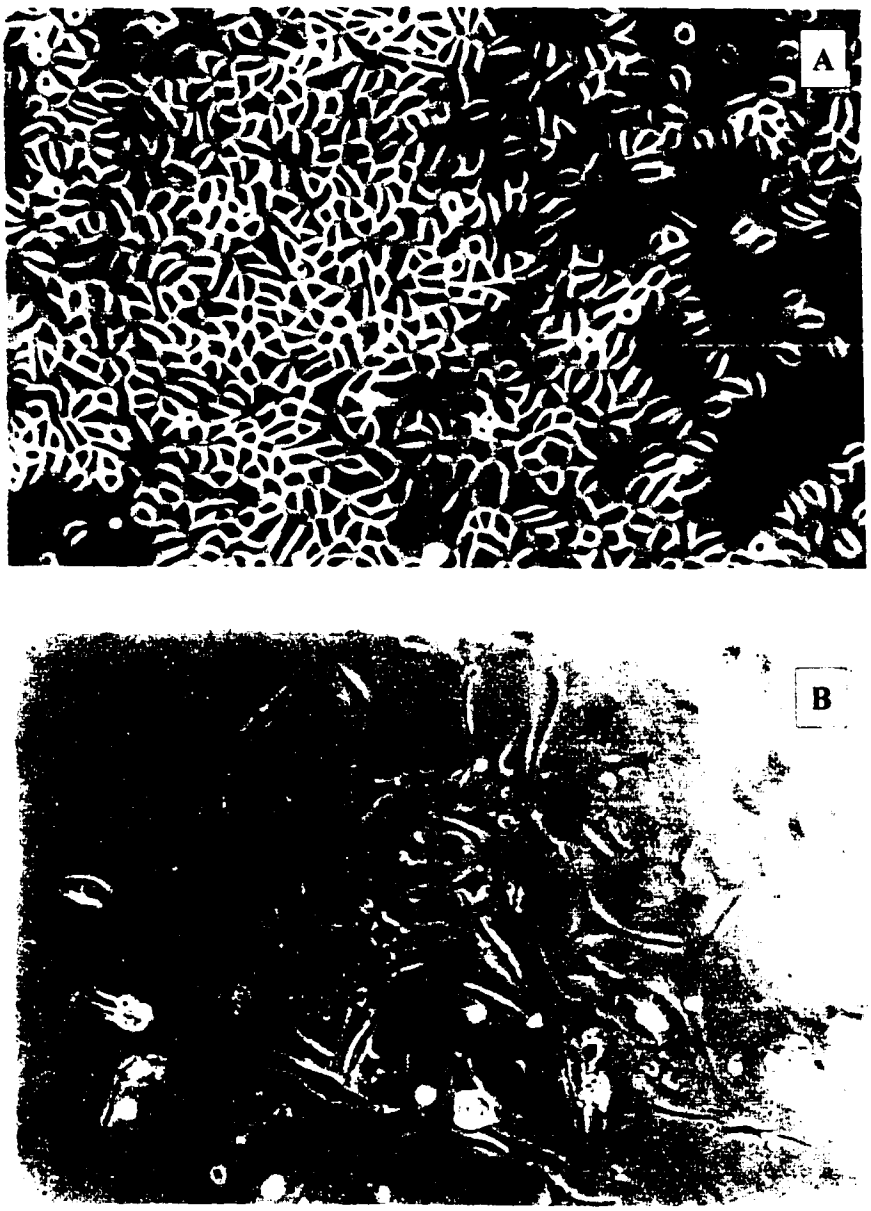


Figure 1.5 Phase-contrast microscopy of esophageal squamous cells and esophageal fibroblasts. Typical morphology of *A*, established esophageal squamous cells; *B*, esophageal fibroblasts in primary culture.

Chapter 2: Characterization of Cultured Barrett's Esophagus Cells

Introduction

Genetic instability is a hallmark of neoplastic progression in Barrett's esophagus, and the progression to cancer has been shown to involve an accumulation of genetic and cell cycle abnormalities (Neshat, 1994a; Reid, 1996; Barrett, 1999). Cell cycle and flow cytometric DNA content abnormalities that occur in the development of cancer in Barrett's esophagus include a mobilization of cells from G_0 to G_1, followed by an increase in 4N (G_2/tetraploid) fractions (Reid, 1991; Reid, 1992; Reid, 1993). In addition, a number of molecular genetic abnormalities have been shown to be involved in the pathogenesis or progression of Barrett's adenocarcinomas, including non-random loss of heterozygosity (LOH) involving five chromosome arms (5q, 9p, 13q, 17p and 18q) (Barrett, 1996a). In particular, 17p and 9p21 LOH occur as early events during the progression to cancer in Barrett's esophagus and are associated with the inactivation of cell cycle regulatory proteins, p53 and the cyclin dependent kinase inhibitor CDKN2/p16 (also called INK4a, MTS1 or CDK4I) (Reid, 1996; Neshat, 1994b; Blount, 1994; Barrett, 1996b). Inactivation of p53 by 17p LOH and mutation of the remaining allele develops in diploid (2N) cells of premalignant Barrett's epithelium and is interdependent with the development of increased 4N (G_2/tetraploid) fractions (Neshat 1994b; Blount, 1994). Elevated 4N (G_2/tetraploid) fractions are strongly predictive of the subsequent development of aneuploidy and cancer (Galipeau, 1996; Reid et al, submitted).

We have isolated Barrett's epithelial cells from endoscopic biopsies obtained from patients with Barrett's esophagus and have successfully grown these cells in culture for twelve to seventeen months. Some of the most common cell cycle and molecular genetic abnormalities previously identified in biopsies from patients with Barrett's esophagus are present in these cultures. In addition, the cultured cells exhibit cytogenetic and ploidy abnormalities. We find that tetraploid cells are present in the Barrett's epithelial cultures and that in at least one case, some of the abnormalities found in the tetraploid component are the same as those found in the diploid component. This suggests that the tetraploid cells were generated from a diploid precursor present in the culture. The establishment of long-term Barrett's cell cultures that contain cell cycle and molecular genetic abnormalities that are

associated with cancer in Barrett's esophagus *in vivo* provides a clinically relevant *in vitro* premalignant model in which to test potential therapies for Barrett's esophagus, as well as examine etiologic factors and genetic intermediates important in neoplastic progression.

Results

DNA content analysis

We established four long-term Barrett's esophagus cultures (CP-52731; CP-18821; CP-94251; KR-42421) from endoscopic biopsies as described previously (Chapter 1). Flow cytometric analysis at early passage showed that all four cultures contained significant proportions of cells with a 4N DNA content (Figure 2*A-D*). The approximate percentage of cells with a 4N DNA content for each cell line was 24.9% for CP-52731, 19.8% for CP-18821, 22.8% for CP-94251, and 11.6% for KR-42421. The histograms from all four cultures indicated the possibility that, in addition to a cycling diploid (2N) cell population, a cycling tetraploid (4N) cell population was also present. To explore this possibility, the 4N subpopulation of cells from two of the cultures (CP-18821 and CP-52731) were viably sorted, returned to culture after sorting and left undisturbed for up to 5 days. For CP-18821, approximately 50% of the cells in the original sorted 4N population were in a diploid cell cycle, with the remainder of cells in a tetraploid cell cycle, based on cell cycle distribution analyses using the Multiplus software program (Figure 2*E-F*). Similar results were obtained from the sorting experiment using CP-52731 (data not shown). Fluorescence *in situ* hybridization of DNA flow sorted 4N cells using centromeric chromosome probes in subsequent studies showed that tetraploid cells were present in the 4N fraction (see Chapter 4).

Cytogenetic analysis

Cytogenetic analysis of mitotic cells was performed on each cell line at one passage level. Late passage cells were used from cultures CP-94251 and KR-42421 and middle passage cells were used from cultures CP-52731 and CP-18821. Chromosome counts of mitotic cells from the cell cultures showed either hypodiploid or hyperdiploid modal chromosome numbers: CP-94251 (44); CP-52731 (44); KR-42421 (50); CP-18821 (41). The chromosome numbers do not reflect the actual DNA

content in the cells because chromosome rearrangements were also present. Two of the cell cultures (CP-94251; KR-42421) showed dicentric chromosomes in at least two mitotic cells examined. In addition to dicentric chromosomes, ring chromosomes were also noted in one of the cell cultures (KR-42421). Thus, each of the cultures examined is peridiploid; the flow cytometric results suggest that the total DNA content change is small (within 10% of diploid). Cells with near-tetraploid chromosome numbers were also observed in all four cultures.

Karyotyping of one of the Barrett's cultures (CP-52731) at middle passage showed that, in the mitotic cells examined, clonal abnormalities present in the near-diploid cells were also present in at least one near-tetraploid cell examined (Figure 3). These included a translocation on 9p, a 17p abnormality, and an unidentified marker chromosome. This suggested that the near-tetraploid cells present in the culture evolved from the near-diploid population, possibly due to missed cell cycle checkpoints. Spontaneous generation of tetraploid cells from diploid precursors was found to occur in later studies (Chapter 3). More detailed cytogenetic studies, including spectral karyotyping, were subsequently performed on all four of the Barrett's cell strains as part of studies involving transduction of the reverse transcriptase subunit of human telomerase (hTERT) (see Chapter 4).

Allelic losses in vitro and in vivo

The following data was obtained in collaboration with Michael T. Barrett and Patricia C. Galipeau, as previously published (Palanca-Wessels, 1998). Molecular genetic analysis was performed on the Barrett's cultured cells in order to assess the occurrence of LOH at the five chromosome arms (5q, 9p, 13q, 17p, and 18q) that are lost non-randomly in Barrett's esophagus *in vivo* (Barrett, 1996). For each culture, DNA from sorted 2N, 4N, and 8N populations was analyzed separately for LOH. For all *in vitro* molecular genetic analyses, DNA samples from at least two different serial passages (early, mid- or late passage) from each culture were examined. Results from the molecular genetic analysis of the different passages from each culture did not differ, indicating that the pattern of LOH from each culture at these particular sites remained stable over time. Chromosome instability, however, was

found using FISH centromeric probes directed at chromosomes only one of which (chromosome 18) was assessed by microsatellite analysis (see Chapter 4).

17p LOH was identified in all cultures except for KR-42421. 9p LOH was detected in three of the cultures (CP-52731; CP-94251; KR-42421) in all sorted samples examined. For the remaining culture, CP-18821, a homozygous deletion involving the p16 intragenic STS marker C5.1 was detected in the 4N and 8N sorted samples examined. 5q LOH was found only in the 4N and 8N sorted samples of KR-42421. No LOH involving 13q or 18q was detected in any of the cultures (Table 2.1).

For each patient, DNA samples from flow-sorted *in vivo* biopsies spanning the entire Barrett's segment were also analyzed. In most cases where *in vivo* biopsies were available from the appropriate level used for tissue culture, the same abnormalities were detected in both tissue culture and patient samples. These included 17p LOH in patients 94251 and 52731 and 9p LOH in patients 94251 and 18821. One exception was patient 18821 in which 17p LOH was detected in each *in vitro* sample (Table 2) but was not detected in the *in vivo* samples.

p53 and p16/CDKN2 mutations

p53 mutations were detected in three cell strains (CP-52731, CP-94251 and CP-18821) in at least one flow sorted diploid (2N) and at least one tetraploid (4N) fraction from each patient. Two of the mutations were single base pair changes (G to A transition in codon 175 and a C to T transition in codon 248). The third cell strain (CP-18821) contained a single base pair deletion within codon 302 (Exon 8) that results in frameshift mutation. In each case, DNA samples from normal gastric mucosa from each patient was wild-type, indicating that the mutations are not of germline origin. For the fourth cell strain (KR-42421), *p53* exons 5-8 were sequenced in 2N and 4N fractions from middle passage cells with no mutations identified (Table 2).

DNA samples from *in vivo* biopsies from each patient were also sequenced for *p53* mutations. For the three cell strains in which mutations were identified, the exon that harbored the mutation was sequenced using DNA from flow sorted *in vivo* samples. In two patients (52731 and 94251), the same *p53* mutation that was present in the corresponding cell strain was also identified in *in vivo* samples. In the third patient (18821), in which a single base pair deletion was identified in exon 8 of the cell strain, no mutation was found in ten sorted samples from five biopsies spanning the length of the patient's Barrett's segment. It is of note that these *in vivo* biopsies from patient 18821 retained both 17p alleles, whereas the cell culture derived from this patient's biopsies contained a p53 mutation. For one patient (42421), we detected no *p53* mutations within exons 5-8 in both cell strain and *in vivo* biopsy samples.

p16/CDKN2 abnormalities were detected in three of the cultures (CP-52731, CP-18821, and KR-42421). For CP-52731, a missense mutation (leucine to glutamine in codon 97) was found in all flow sorted samples examined. Furthermore, deletion of the C5.1 STS marker in the *p16* gene was found in both CP-18821 and KR-42421. For CP-18821, the deletion in *p16* was detected in both 4N and 8N flow sorted samples, whereas for KR-42421 the deletion was detected only in the 8N sorted sample. Sequencing of the *p16* gene was performed on *in vivo* biopsies from the two patients with 9p21 LOH (94251 and 18821). A missense mutation (histidine to tyrosine in codon 83) was found in flow sorted *in vivo* samples from patient 94251 whereas there was no mutation detected in patient 18821. In both cases, however, the *in vivo* biopsies used for *p16* sequencing were obtained from a level in the Barrett's segment different from the level from which the biopsies for tissue culture were obtained.

Discussion

The cell cultures that have been established and described in this paper are the first long-term Barrett's epithelial cell cultures shown to possess molecular genetic defects that are commonly found in premalignant Barrett's esophagus *in vivo* (Reid, 1991); these include LOH at 5q, 9p, 13q, 17p and 18q chromosome arms and *p53* and *CDKN2/p16* abnormalities. Most striking is the observation that the cell cycle and molecular genetic abnormalities found in the subset of biopsies that gave rise to long-

term *in vitro* cultures are also found early in the sequence of events leading to cancer in Barrett's esophagus *in vivo*. Elevated 4N fractions, 17p LOH and *p53* inactivation, and 9p21 LOH with and without *CDKN2/p16* mutation can be found early in progression to adenocarcinoma (Neshat, 1994a; Blount, 1994; Barrett, 1996b; Galipeau, 1996). These abnormalities are also present in the Barrett's epithelial cultures. All four cultures contain elevated 4N fractions and 9p21 and/or *CDKN2/p16* abnormalities. Inactivation of *p53*, by 17p LOH and mutation in the remaining allele, is also present in three of the four cultures. This suggests that some of the genetic and cell cycle alterations that precede the development of cancer *in vivo* also allows Barrett's cells to grow *in vitro*. Therefore, the study of the cultured cells' response to potential growth stimulatory agents present in Barrett's esophagus may provide clues to the behavior of Barrett's epithelial cells *in vivo*.

In general, the molecular genetic abnormalities detected in an *in vitro* culture were the same as those present in the patient from whose biopsies the culture was derived. There were a few exceptions where the *in vitro* and *in vivo* results differed. However, because the Barrett's segment *in vivo* can be composed of a mosaic of different clonal populations (Barrett, 1999; Rabinovitch, 1988; Raskind, 1992), one possibility is that the cells that became established in culture were derived from a small clone within the patient's Barrett's segment that was missed or not detected in the *in vivo* samples. This is likely since *in vitro* culture conditions necessarily select for the clone that proliferates best in those conditions, even though that particular clone may be small *in vivo*. An indication that a genetic alteration(s) is required for continuous proliferation in culture is provided by the observed statistical association between *in vivo* presence of 17p LOH and successful establishment of long-term cultures (see Chapter 1). The development of improved primary culture techniques, however, appears to increase the permissibility for growth of cells containing normal p53 (see Chapter 1).

It has been previously shown that increased 4N fractions in patients with Barrett's esophagus are a strong predictor for the subsequent development of aneuploidy and cancer (Galipeau, 1996). DNA flow cytometric analysis shows that all four cell cultures have an elevated 4N fraction that is partly comprised of tetraploid G_1 cells, as indicated by cell sorting experiments and cytogenetic analyses

(see also Chapters 3 and 4). We were able to detect, by microsatellite analysis, genetic abnormalities in 4N and 8N fractions, suggesting that these abnormalities were only present in the cycling tetraploid fraction. We found subsequent to these experiments, however, that the tetraploid population appears to be unstable and is continually being regenerated from the diploid cells *in vitro* (see Chapter 3). An explanation for our findings is that there may be a different relative enrichment for cells containing a particular genetic change in the 2N, 4N, and 8N fractions of the culture. The allelic loss assay is PCR-based and is sensitive to the proportion of cells containing a particular LOH. Thus, there may be cells present in the 2N fraction that contain an LOH, but not at a high enough percentage to score it as a loss.

Serial examination of genetic alterations in successive passages *in vitro* by microsatellite analysis did not show evidence of evolution, and all cultures senesced after 17 to 29 population doublings. This suggests that the rate of genetic evolution *in vitro* is probably slow, and that the large cell numbers involved in Barrett's esophagus field defects and growth aover the span of years are needed to allow accumulation of rare events in neoplastic evolution. Moreover, other factors not present *in vitro* may hasten the development of aneuploidy and immortalization *in vivo*. For example, conditions present in Barrett's esophagus *in vivo* (e.g. bile acids or nitrosamines (Mirvish, 1993; Pera, 1993)) may challenge defective cell cycle checkpoint(s) leading to the development of aneuploidy in Barrett's epithelial cells lacking *p53* function. The cell cultures could be used to test such hypotheses. In addition, because the cultures are comprised of a mixture of both diploid and tetraploid cells, it may be possible to determine whether tetraploid cells are more genetically unstable and predisposed to neoplastic progression (see Chapter 3), thus explaining the observation *in vivo* that elevated 4N fractions precede the development of aneuploidy (Galipeau, 1996). None of the Barrett's cultures have spontaneously developed the flow cytometric aneuploidy characteristic of more advanced stages of neoplastic progression in Barrett's esophagus (Rabinovitch, 1988), despite elevated 4N fractions and inactivation of *p53*, or given rise to immortal cultures. Activation of telomerase, however, is a plausible mechanism by which immortalization of the Barrett's cultures and further evolution could occur. We subsequently investigated this in Chapter 4.

Karyotyping of CP-52731 shows that the tetraploid cells possess some of the same chromosome abnormalities found in the diploid cells suggesting that the tetraploid cells in this culture developed by a process of clonal evolution from a diploid progenitor cell (see Chapter 3). It has been suggested previously that inactivation of *p53* in Barrett's esophagus and colon cancer predisposes to tetraploidy as a consequence of inactivation of cell cycle checkpoints and endoreduplication (Galipeau, 1996; Carder, 1993). The frequent occurrence of tetraploid cell populations in *p53*-/- cultured Barrett's epithelial cells and the cytogenetic evidence for evolution of a tetraploid cell population from a diploid *p53*-/- Barrett's epithelial cell *in vitro* provide further support for this hypothesis. Sorting experiments suggest that tetraploid cell formation is an ongoing process (Chapter 3). Interestingly, we have observed numerical centrosome abnormalities in the cultured cells, particularly in the tetraploid component (Chapter 3). Abnormalities in both spindle checkpoint function and centrosome numbers have been attributed to loss of p53 function and predispose to the development of polyploidy after challenge with mitotic spindle inhibitors (Levine, 1991; Cross, 1995; Fukasawa, 1996).

The availability of genetically characterized premalignant cultures for study provides the unique opportunity to investigate those factors that are important in both the progression to cancer and immortalization. *In vitro* studies could also assist in the discovery of effective treatments for Barrett's esophagus. The identification of some of the early cell cycle and genetic changes that are present in pre-dysplastic Barrett's epithelium may allow the rational design of treatments that take advantage of these alterations. Premalignant human cell cultures that contain a combination of cell cycle and genetic abnormalities that lead to cancer *in vivo*, such as increased 4N (G_2/tetraploid) fractions and *p53* and *CDKN2/p16* inactivation, provide a clinically relevant *in vitro* system for the preclinical assessment of potential therapeutic agents (Chapter 5). Because the Barrett's epithelial cultures contain a variety of defined molecular genetic defects, differences in response between cultures can be correlated to differences in genotype. Since p53 inactivation occurs early in progression to cancer in Barrett's esophagus, strategies which selectively remove cells lacking normal p53 function may be

potentially useful in the treatment or prevention of cancer in this condition (Bischoff, 1996; Fan, 1995; Hawkins, 1996; Powell, 1995; Russell, 1995).

Methodology

Cell culture maintenance

Barrett's esophagus cell strains (CP-18821; CP-52731; CP-94251; KR-42421) were maintained in MCDB 153 containing growth supplements as previously described (Chapter 1; Palanca-Wessels, 1998). Normal human diploid fibroblasts (82-6; LG EF) were grown in DMEM containing 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin.

Cytogenetics

Colcemid (Gibco, Grand Island, NY), 0.2 μg/ml, was added 24 to 37 hours prior to harvest for cytogenetic analysis. Cells were then incubated in hypotonic solution (potassium chloride and sodium citrate dihydrate) for 20 minutes at 37°C prior to being fixed in methanol: glacial acetic acid (3:1). Slides were prepared and G-banded according to standard cytogenetic methods. Modal chromosome numbers were established by recording the number of chromosomes in each mitotic cell in which all chromosomes could be reliably counted; at least 50 cells were counted for each culture to establish the modal number. Structural chromosome abnormalities were considered clonal if the alterations were exhibited in at least 2 cells from a culture.

Flow cytometry

DNA content flow cytometric sorting was used to purify different ploidy populations from both *in vivo* and *in vitro* samples using a Coulter Elite cell sorter (Miami, FL). For *in vivo* samples, biopsies were processed before sorting as described previously (Neshat, 1994b; Blount, 1994). For viable sorting of cultured cells, cells were incubated in growth medium containing Hoescht 33342 (Calbiochem, La Jolla, CA) at 10 μM concentration for 30 minutes at 37°C. Cells were trypsinized after incubation, resuspended in new growth media containing dye, and kept covered at room temperature until sorting. For DAPI staining, cultured cells were trypsinized and resuspended in 10

μg/ml DAPI in NST buffer [146 mM NaCl, 10 mM Tris base, 0.1% NP40 (Sigma), 2 mM Ca, 20 mM Mg, and 0.05% BSA, pH 7.4] before analysis or sorting. The Multiplus software program (Phoenix Flow Systems, San Diego, CA) developed by Dr. Peter S. Rabinovitch was used for cell cycle analysis.

Microsatellite Analysis

DNA was extracted using standard SDS/proteinase K and phenol/chloroform methods (Blount, 1994). To screen multiple loci in small, highly purified flow sorted samples in polymerase chain reaction (PCR) assays, DNA was amplified by the technique of whole genome amplification (Zhang, 1992; Barrett, 1995). Briefly, DNA corresponding to 1000 cells from each sample was randomly amplified using a mixture of degenerate 15-mers to prime the reaction in a 60 μl volume according to published protocols (Zhang, 1992). Aliquots of whole genome amplified DNA were evaluated for polymorphic markers in locus-specific PCR assays. A series of polymorphic markers was used to screen the known high frequency sites of LOH in Barrett's adenocarcinoma (Barrett, 1996a). The markers used were *D5S107, D5S299, D9S942, D9S43, D13S314, D17S5, TP53 ALE3, D17S1176, D18S46, D18S34,* and *D18S474.* Reagents were used as described previously with the addition of [γ-^{32}P]adenosine triphosphate (ICN, Irvine, CA) T4 polynucleotide kinase (New England Biolabs, Beverly, MA) end-labeled primer (Barrett, 1996a). PCR products were run on denaturing 6% polyacrylamide gels, vacuum dried, and then exposed to X-ray film.

DNA Sequence Analysis

Template for DNA sequence analysis was obtained using DNA from 10,000 normal or flow-sorted cells according to our published methods (Neshat, 1994b; Blount, 1994). Primers for amplifying and sequencing *p53* and *CDKN2/p16* were based on published sequences (Neshat, 1994b; Kamb, 1994). Template for sequencing *CDKN2/p16* was obtained by heminested PCR as previously described (Barrett, 1996b). Products were purified then used directly in sequencing reactions as described elsewhere (Galipeau, 1996).

All PCR reactions were performed with a MJ Tetrad thermal cycler (MJ Research Corp.,UK). All samples were sequenced by automated sequencing with fluorescently labeled dideoxy chain-terminating nucleotides and Taq DNA polymerase according to the suppliers specifications (Applied Biosystems, Inc., Foster City, CA). The products were analyzed on an Applied Biosystems Model 373 Automated DNA sequencing machine as previously published (Neshat, 1994b). Constitutive DNA was evaluated by the same method to confirm that mutations were somatic.

Table 2.1 Allelic Losses and Mutations Found Barrett's Epithelial Cell Cultures

The allelic losses and mutations were found in 2N, 4N, and 8N sorted DNA samples unless otherwise noted. *Codon numbering based on revised *p16* gene sequence (Hannon, 1994; Quelle, 1995). Abbreviations: C5.1, STS marker; Del, deletion; NI, non-informative for marker.

	5q	*13q*	*18q*	*9p*	*CDKN2/p16*	*17p*	*p53*
CP-52731	2	NI	1 (4N,8N)	1	Leu→Gln 97	1	Arg→His 175
CP-94251	2	2	2	1	Wild-type	1	Arg→Trp 248
CP-18821	2	2	2	Del (C5.1) (4N,8N)	Del (C5.1) (4N,8N)	1	Frameshift 302
KR-42421	1 (4N,8N)	2	2	1	Del(C5.1) (8N)	2	Wild-type

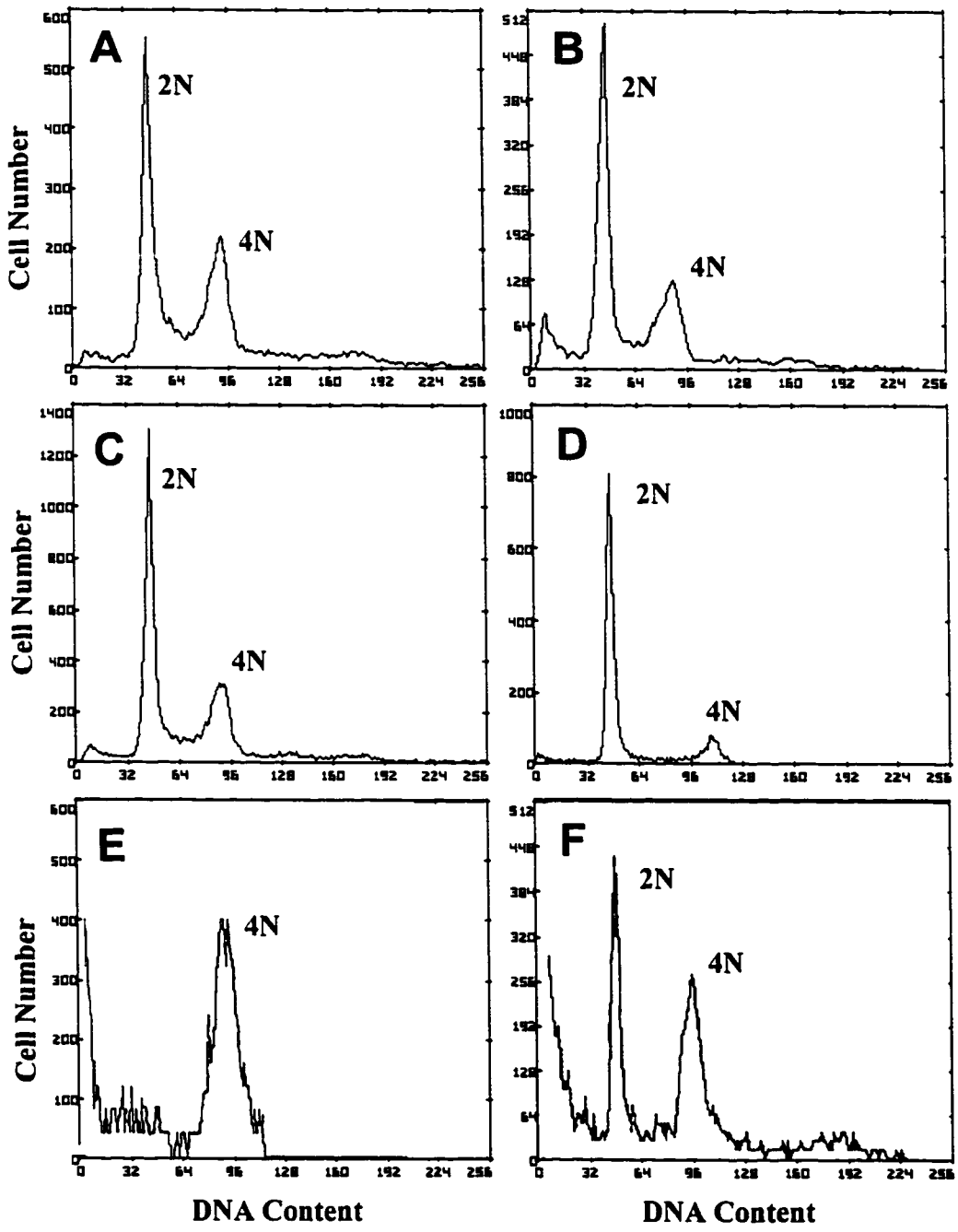


Figure 2.1 Flow cytometric analyses of asynchronous and 4N sorted Barrett's epithelial cultures. Cell cycle distribution of asynchronous cells are shown in *A-D*. Note the elevated fraction of cells with G_2/tetraploid (4N) DNA content. *A*, CP-52731; *B*, CP-18821; *C*, CP-94251; *D*, KR-42421. DNA histograms of 4N sorted cells from one of the Barrett's epithelial cultures, CP-18821, are shown in *E* and *F*. 4N sorted cells from CP-18821 were returned to culture and harvested 72 hours later. Sorted 4N cells: *E*, immediately after sort; *F*, 72 hours later.

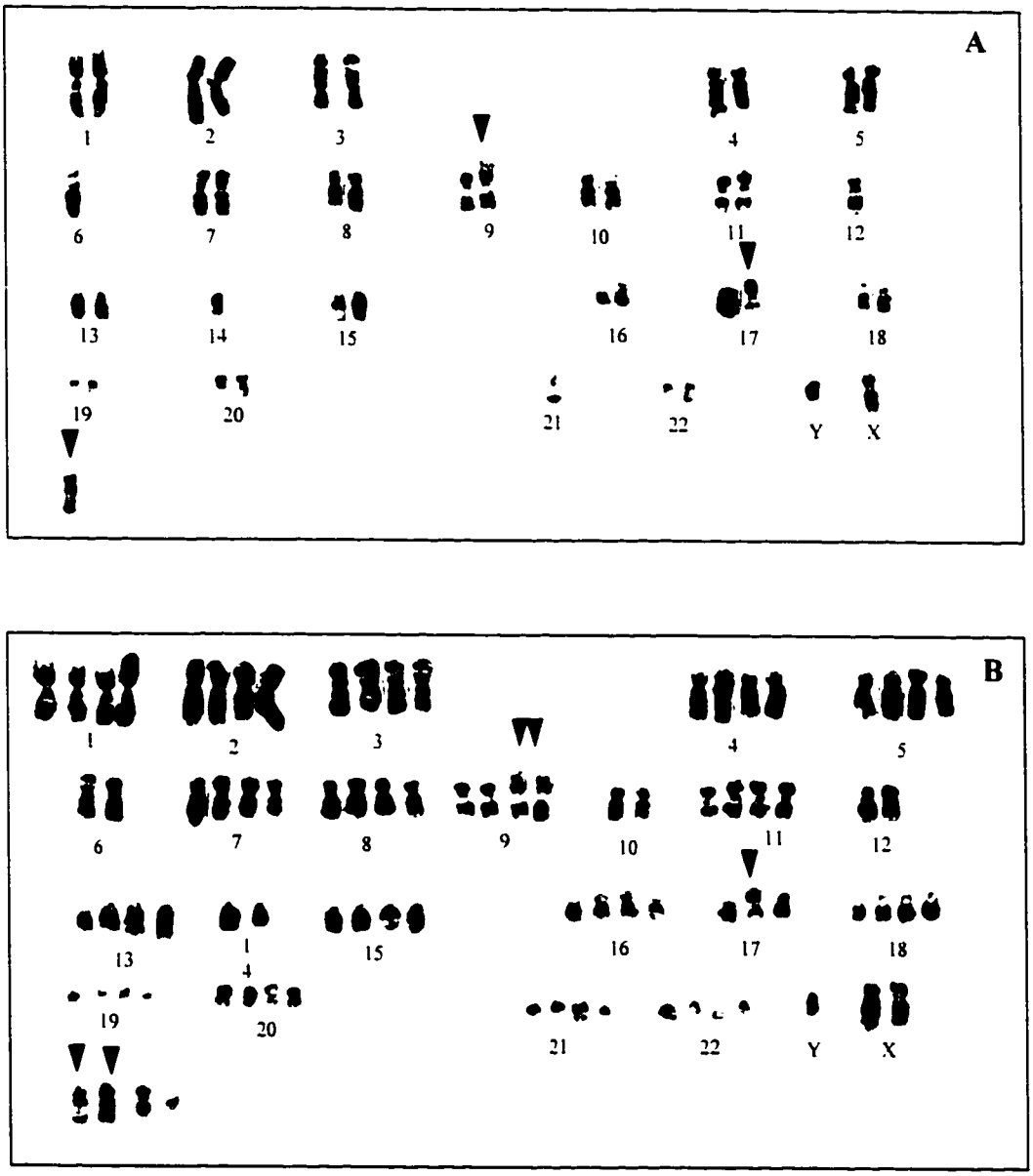


Figure 2.2 Karyotypes of a near-diploid cell, A, and a near-tetraploid cell, B, from culture CP-52731. Clonal abnormalities found in the near-diploid cell which are also seen in the near-tetraploid cell include a translocation onto the 9p chromosome, a 17p abnormality and an unidentified marker chromosome (designated by arrowheads).

Chapter 3: Genomic Instability in Barrett's Esophagus Cells

Introduction

Neoplastic progression is characterized by increased genomic instability that may be manifested in numerous ways, including DNA microsatellite instability, gene amplification, and karyotypic abnormalities. In normal cells, several mechanisms exist that prevent the propagation of genetic errors during the course of cell growth and division. DNA repair pathways correct errors that occur during the process of DNA replication or mend damaged DNA. In addition, cell cycle checkpoints ensure the orderly progression through the cell cycle by making the transition from one cell cycle phase to another dependent on the completion of prior events. For example, mitotic entry is blocked unless S phase replication is finished. Similarly, re-replication of DNA is prevented unless successful completion of mitosis has occurred. Defects in cell cycle checkpoint function can result in the genetic instability seen in tumors. Not surprisingly, the tumor suppressor gene p53 which participates in a number of cell cycle checkpoints is commonly mutated in a variety of human neoplasms (Hollstein, 1991).

Aneuploidy is frequently seen in human cancers and may arise as a result of cell cycle checkpoint deficiencies. The fidelity of chromosome transmission depends upon the correct assembly and orientation of the mitotic spindle leading to the accurate segregation of chromosomes. Treatment of cells with antimicrotubule agents such as nocodazole and colcemid blocks the assembly of the mitotic apparatus and prevents the cell from undergoing normal mitosis and cytokinesis. After a period of mitotic arrest, the cell adapts and reverts to a G1-like state, still containing a tetraploid 4N DNA content, a process commonly referred to as endoreduplication. Normal cells are unable to subsequently progress further through the cell cycle (Lanni, 1998; Khan, 1998). However, mutations in tumor suppressor genes, such as p53 and pRb, abrogate this checkpoint leading to a proliferating tetraploid cell cyle, as shown in Figure 3.1 (Cross, 1995; Di Leonardo, 1997; Lanni, 1998; Khan, 1998). Survival of resulting tetraploid cells may be dependent on the expression of apoptosis suppressing genes such as Bcl-xL and the successful completion of the subsequent mitosis (Minn, 1996).

Tetraploid cells produced from a missed checkpoint may represent an unstable population from which aneuploid cells may develop by loss of genetic material (Shackney, 1989). Tetroploidy, with a doubling of the normal number of chromosomes, may increase the likelihood of misalignment at the metaphase plate and lead to unequal chromosome segregation due to lagging chromosomes. Alternatively, endoreduplication to tetraploidy also leads to amplification of centrosomes (Figure 3.1); the presence of more than two centrosomesat mitosis can give rise to aberrant multipolar mitoses that produce aneuploid cells (Brinkley, 1998). Polyploid cells of the budding yeast *Saccharomyces cerevisiae* contain higher levels of chromosome loss (Mayer, 1990). Tetraploidization of cultured Chinese hamster ovary cells after drug treatment induces genetic instability and the cells rapidly become aneuploid (Andreassen, 1996). In a study of elastase-SV40 tumor antigen transgenic mice, the appearance of tetraploid cells in pancreatic tissues coincided with the appearance of cells possessing abnormal centriole numbers (Levine, 1991). In this mouse model, tumor formation follows a diploid→ tetraploid→aneuploid sequence suggesting that tetraploid cells represent a genetically unstable intermediate. Since SV40 tumor antigen inactivates both p53 and pRb function, centrosome abnormalities could arise due to a deficiency in cell cycle checkpoints involving these tumor suppressors.

Recently, there has been much renewed interest in the involvement of centrosomes in neoplastic progression, an idea originally put forth by Theodor Boveri (Boveri, 1929; Brinkley, 1998). The centrosome represents the microtubule organizing center in animal cells and consists of two centrioles surrounded by pericentriolar material that primarily consists of γ-tubulin and pericentrin (Zheng, 1995; Doxsey, 1994). The centrosome undergoes a replication cycle which parallels DNA replication and begins near the G1-S phase transition leading to two centrosomes in G2-M phase that separate and form the spindle poles during mitosis. In mice, inactivation of p53 or amplification of MDM2 can lead to centrosome amplification, chromosome instability and aneuploidy (Fukasawa, 1996; Fukasawa, 1997; Wang, 1998; Carroll, 1999). Most recently, murine embryonic fibroblasts containing mutated BRCA1 have also been shown to contain centrosome abnormalities (Xu, 1999).

Centrosome abnormalities, both structural and numerical, have been observed in both human tumors and tumor cell lines (Lingle, 1998; Pihan, 1998; Weber, 1998; Sato, 1999). Inactivation of p53 by mutation or MDM2 overexpression in human tumors has been associated with centrosome abnormalities (Carroll, 1999). Overexpression of centrosome related proteins such as BTAK (aurora2) and pericentrin have also been linked to centrosome amplification (Purohit, 1997; Zhou, 1998; Brinkley, 1998; Bischoff, 1998).

While these studies have shown that centrosome abnormalities are present in murine and human cancers, they have not examined the relationship between polyploidization and centrosome abnormalities in *premalignant* human cells. Centrosome amplification in tetraploid cells at an early stage in tumor progression may enhance the rate of genetic evolution by providing a mechanism for loss or gain of chromosomes that may, on rare occasion, lead to the generation of a viable, malignant, aneuploid cell (Brinkley, 1998). Barrett's esophagus is a human preneoplastic condition in which the normal squamous epithelial lining of the esophagus is replaced by a metaplastic columnar epithelium that is at high risk for the development of esophageal adenocarcinoma (Neshat 1994a; Reid, 1996; Jankowski, 1999). Flow cytometric analyses of Barrett's esophagus patient biopsies have shown that, in general, elevation of 4N (G2/tetraploid) DNA content fractions (defined as >6% of the total cells) are observed concomitantly with p53 inactivation and predispose to the development of aneuploidy (Galipeau, 1996). Aneuploidy is observed, on average, approximately 17 months after the initial detection of elevated 4N fractions (Galipeau, 1996). Patients with either elevated 4N fractions or aneuploidy are at increased risk for subsequent development of cancer compared to patients who do not have these flow cytometric abnormalities at initial evaluation (Reid et al, submitted). These data suggest that the 4N population is an important intermediate in the development of aneuploidy and cancer.

We have established four Barrett's esophagus strains from precancerous tissue that all showed elevated 4N (G2/tetraploid) fractions which were subsequently found to contain tetraploid cells by karyotypic analysis (Chapter 2 and Palanca-Wessels, 1998). Three of the cell strains (CP-52731; CP-

94251; CP-18821) contain p53 mutations (Chapter 2 and Palanca-Wessels, 1998). The remaining cell strain (KR-42421) contains no 17p loss of heterozygosity and a wild-type p53 gene sequence, but p53 function appears to be compromised based upon evidence of loss of the p53-dependent G1 cell cycle checkpoint, which was manifested by lack G1 arrest and continued bromodeoxyuridine uptake (S phase entry) after irradiation (Palanca-Wessels, unpublished results). Murine $p53^{-/-}$ fibroblasts polyploidize after treatment with microtubule inhibitors due to abnormalities in cell cycle checkpoints (Cross, 1995) and also show centrosome amplifcation (Fukasawa, 1996). We wanted to determine whether centrosome abnormalities were present in untreated Barrett's esophagus cultures. If tetraploid cells possess abnormal centrosome numbers, this may contribute to the apparent instability of the 4N fraction and the subsequent formation of aneuploid cells *in vivo*. Abnormalities in the diploid component, if present, might suggest that centrosome disregulation occurs in the diploid cell cycle and that diploid G2/M cells are the initial genetically unstable cells in the 4N fraction. In order to determine which ploidy population might contain centrosome amplification, we use flow cytometry to sort cells on the basis of ploidy and then stained these sorted cells for pericentrin, a component of the pericentriolar material in centrosomes. We found that pericentrin abnormalities increased with ploidy. To determine which cells in the 4N fraction (diploid G2 versus tetraploid G1) contained centrosome abnormalities, we utilized cyclin B expression to differentiate these two populations. We found centrosome abnormalities in both the diploid G2 and tetraploid G1 cells. Furthermore, cells with centrosome abnormalities arose rapidly from sorted diploid cells and this was accompanied by spontaneous tetraploidization in the Barrett's esophagus cell cultures. Finally, we showed that tetraploid cells and pericentrin abnormalities also exist in biopsies from Barrett's esophagus. These results suggest that centrosome abnormalities may be a cause of tetraploidy and chromosome instability in Barrett's esophagus.

Results

Pericentrin amplification correlates with increasing ploidy in cultured Barrett's esophagus cells

As described in Chapter 2, cultured Barrett's esophagus cells have an elevated proportion of 4N cells present in culture (11.6% to 24.9% of total cells). We used flow cytometry to sort each of the four

Barrett's esophagus cell strains by ploidy (2N, 4N, and 8N DNA content) on two separate occasions. 2N and 4N fractions of one normal diploid human fibroblast strain (82-6) were also sorted as a control. After sorting on the basis of DNA content, the viable cells were allowed to adhere to glass slides during a five hour incubation prior to staining with an antibody directed against pericentrin, a protein component of the pericentriolar material in centrosomes. Normally, diploid cells possess either one pericentrin focus or two closely spaced pericentrin foci until the onset of mitosis, when the two foci separate and the centrosomes migrate to form the poles of the mitotic spindle (see Figure 3.1). The distribution of pericentrin foci in the various sorted ploidy populations in the human fibroblast and Barrett's cell strains are shown in Figures 3.2 through 3.6. In the normal human diploid fibroblast strain 82-6, the 2N sorted fraction contained either one focus or two closely spaced pericentrin foci (Figure 3.2). These represent either cells in G1 (one focus) or cells that have started to traverse through S phase and have begun replication of the centrosomes (two close foci). The 4N sorted fraction contained a large proportion of cells with two pericentrin foci, either in close proximity (2C) or widely separated (2S); these cells have replicated their centrosomes, as shown in Figure 3.1. Approximately 30% of the 82-6 cells present in the 4N fraction contained only one pericentrin focus. These represent either: 1) diploid cells that have completed mitosis and re-entered G1 during the five hours in which the sorted cells were allowed to adhere to slides; 2) a staining artifact due to poor antibody detection or overlapping pericentrin foci; or 3) tetraploid cells with missegregation of centrosomes. The first two of these possibilities are the most likely and, in general, the normal human diploid fibroblasts contained normal numbers of pericentrin foci, as expected.

In the Barrett's esophagus cell strains, however, pericentrin amplification (defined as greater than two pericentrin foci) was consistently found to increase with ploidy, although the degree of excess foci varied between cell strains (Figures 3.3 through 3.6). The majority of 2N sorted Barrett's cells contained either one or two pericentrin foci, with a few exceptions. The cell strain CP-18821 showed amplified pericentrin foci in the 2N sort, approximately 10% of cells contained three or more foci (Figure 3.6), while 5% of 2N sorted KR-42421 cells contained three or more foci (Figure 3.5). In the same two Barrett's cell strains (KR-42421 and CP-18821), between 12% and 20% of the 2N sorted

cells, respectively, contained two separated pericentrin foci. In all four of the Barrett's cell strains, the 4N and 8N sorted fractions contained increasing proportions of cells exhibiting amplified pericentrin foci. In addition to numerical changes, structural abnormalities such as large and irregularly shaped pericentrin foci were also seen more frequently in higher ploidy cells of the Barrett's cultures. Loose clusters of pericentrin foci were also observed within cells. In contrast, normal fibroblasts rarely contained more than two pericentrin foci and showed no structural abnormalities. Examples of pericentrin staining in both cultured normal human diploid fibroblasts and Barrett's esophagus cells are shown in Figure 3.7.

Pericentrin amplification could occur as a consequence of endoreduplication as depicted in Figure 3.1. Based on this model, the adaptation of cells after mitotic arrest and the subsequent re-entry of these cells into a tetraploid cycle could lead to a doubling of centrosome number. In order to determine whether the observed increase in centrosome number with ploidy was consistent with this process, we plotted the distribution of cells from each ploidy fraction containing: A) the expected number of centrosomes if the endoreduplication model was correct; B) fewer than the expected number of centrosomes; C) greater than the expected number of centrosomes. Each sorted ploidy fraction from the Barrett's cell strains was analyzed separately and the results are shown in Figures 3.8 to 3.10. For 2N cells, one would expect either one or two closely spaced pericentrin foci (Figure 3.8). We found that the majority of cells contained the expected number of foci, but there were some cells that contained greater than the expected number of centrosomes. This varied among the Barrett's strains from 1% to 26% of the 2N sorted fraction. For 4N cells, one would expect two pericentrin foci (either closely spaced or separated). In general, we found that while a large fraction of cells contained the expected number of foci, cells containing fewer than the expected number were almost as large a subset, and in several cell strains there were also appreciable numbers cells containing greater than the expected number of pericentrin foci (Figure 3.9). For 8N cells, we found that the greatest proportion of cells contained fewer than the expected number (four) of pericentrin foci (Figure 3.10). This suggests that there is a selection against cells containing elevated centrosome numbers. The

percentage of 8N cells containing greater than the expected number of foci was a smaller number of cells in this category.

Since elevated 4N fractions *in vivo* are predictive of the subsequent appearance of aneuploidy and cancer, we wanted to carefully investigate the nature of the 4N cells containing amplified centrosomes. If centrosome amplification was solely related to endoreduplication (Figure 3.1), unexpected numbers of centrosomes in the 4N fraction might result from unequal segregation of centrosomes in the 8N mitosis. In this scenario, amplified centrosomes would not be present in 4N diploid G2 cells. In order to differentiate diploid G2/M cells from tetraploid G1 cells in the sorted 4N fraction of the Barrett's epithelial cultures, we used cyclin B as a marker of cells in the late G2/M phase of the cell cycle. In order to prevent diploid cells from completing mitosis, returning to G1 and losing cyclin B expression, we treated 2N and 4N sorted cells with nocodazole after sorting and during cell attachment. Thus, diploid cells in G2/M remained cyclin B positive, whereas tetraploid cells in G1 would be cyclin B negative. After the cells had attached to the slides, the cells were immunostained for both cyclin B expression and pericentrin. The results are shown in Figure 3.11. The percent of diploid G2/M cells (identified by 4N DNA content and high cyclin B expression) containing amplified centrosomes (three or more pericentrin foci) in each of the Barrett's cell cultures was approximately two-fold higher than the percent of cells in the diploid G1 fraction containing amplified centrosomes (see Figure 3.11). Because the proportion of cells containing amplified centrosomes is greater in the diploid G2/M fraction than the diploid G1 fraction, this suggests that a defect in centrosome replication may be present in the Barrett's cell cultures. This has been previously observed by Fukasawa et al in $p53^{-/-}$ mouse embryonic fibroblasts (Fukasawa, 1996). Tetraploid cells with amplified pericentrin comprised one-half to one-third of diploid G2/M cells, the remainder being tetraploid G1 cells (Figure 3.11). For comparison, the percent of 4N cells that were diploid G2/M (cyclin B positive) was approximately 50% in all Barrett's cell strains [CP-52731 47% ± 3% (standard error of the mean); CP-94251 52% ± 3%; KR-42421 51% ± 7%; CP-18821 46% ± 2%].

A dynamic equilibrium exists between diploid and tetraploid cell populations in Barrett's esophagus cells in vitro

We have performed experiments that have demonstrated that tetraploid cells are rapidly generated in untreated Barrett's esophagus cultures and reach an "equilibrium" with diploid cells due to the creation of tetraploid cells that exhibit both lower growth and a higher percentage of apoptosis than diploid cells. We flow sorted Barrett's cells from asynchronous cultures into 2N (diploid G1) and 4N (diploid G2/tetraploid G1) fractions, re-cultured the cell fractions separately, then harvested and re-analyzed the separate fractions by flow cytometry at various times after sorting, as outlined in Figure 3.12. The results of two separate experiments using the Barrett's cell strain CP-52731 are shown in Figure 3.13. If diploid and tetraploid cells were independent and stable cycling populations, sorting of the cells into 2N and 4N fractions should have established cultures that remained enriched for the respective ploidy populations. 2N cells would contain pure diploid G1 cells and 4N cells would contain both diploid G2 and tetraploid G1 cells. After allowing the cells to resume proliferation, we would expect that the 2N (diploid G1) sort would contain a lower percentage of 4N cells than the initial culture because of the removal of tetraploid cells. Likewise, the 4N sort would contain a higher percentage of 4N cells than either the initial culture of the 2N sorted culture because more tetraploid cells would be present and cycling in the 4N sorted culture. We found that this was not the case for the Barrett's cells. The 2N sorted culture showed a rapid rate of gain in the percentage of 4N cells in the culture and approached the percentage of 4N cells present in the original culture within 48 hours after the sort (Figure 3.13 B). The rate of decrease in the percentage of 4N cells in the 4N sorted fraction showed a more gradual asymptotic approach to the original percentage of 4N cells by 15 days post-sorting.

The above results suggested that a steady-state exists between the disappearance of 4N (diploid G2/tetraploid G1) cells from the 4N sort and the generation of 4N (diploid G2/ tetraploid G1) cells from the 2N sort. This equilibrium would require slower growth and/or death of the 4N cells in order to balance their continuous formation from 2N cells. We examined this by flow sorting 2N and 4N cells, re-culturing the ploidy fractions separately, and then comparing the purified fractions for cell

growth and apoptosis. Growth was quantitated by harvesting the cells and counting an aliquot using a hemocytometer. The remaining cells were stained with CMX-rosamine, MitoTracker Green, and Hoechst 33342 fluorescent dyes which permit the differentiation of apoptotic from live cells as well as discriminating DNA content when analyzed on the flow cytometer (Poot, 1999). The experimental protocol is illustrated in Figure 3.14. The results from two Barrett's cell strains (KR-42421 and CP-94251) are shown in Table 3.1. In KR-42421, we found that the 4N sorted culture from the second sort had a lower proliferative capacity and higher apoptotic index than the other sorted cultures. In CP-94251 however, we found that there was no difference in the proliferative capacity of the 2N and 4N sorted cells from the second sort although there appeared to be a higher percentage of apoptotic cells in the 4N sorted culture of the second sort. There were few differences in the proportion of 2N versus 4N apoptotic cells when the DNA content of apoptotic cells within the sorted population was examined.

Generation of tetraploid cells from diploid Barrett's esophagus cells in vitro is accompanied by pericentrin amplification

Since the diploid G1 Barrett's epithelial cells possessed predominantly normal numbers of pericentrin foci but the diploid G2/M cells did not, this suggested that some of the diploid G2/M cells do not go on to generate diploid G1 daughter cells and may convert to tetraploid cells. Tetraploidization would also explain the rapid increase in the percentage of 4N cells in the 2N sorted culture that we observed in the experiments shown in Figure 3.13. We examined whether spontaneous tetraploidization could have accounted for this increase in 4N cells and, if so, whether amplified pericentrin would also be observed in parallel with the increasing 4N (see Figure 3.1). To do this, we sorted viable 2N Barrett's cells and returned them to culture. Sorting only the 2N cells removed any pre-existing tetraploid cells in the culture and ensured that any tetraploid cells subsequently observed were generated from diploid cells. At various times post-sorting, we harvested and re-sorted the cells on the basis of ploidy, again into 2N and 4N fractions for subsequent pericentrin and FISH analyses. An outline of the experimental protocol is shown in Figure 3.15. 4N cells containing three or more pericentrin foci were scored as pericentrin amplified. To determine the percentage of tetraploid cells, we used FISH

centromeric probes to examine the sorted 4N fraction. It has been previously shown that the centromeres of chromosome separate following adaptation to mitotic arrest and thus four centromeric FISH signals are an indication of tetraploid G1 cells (Casenghi, 1999). In our study, only non-mitotic cells possessing four separated FISH signals were scored as tetraploid. 4N cells containing two pairs of closely opposed FISH spots (representing replicated centromeric regions on unseparated sister chromatids) were considered diploid G2 cells and were not counted as tetraploid cells. The results from three Barrett's cell strains and a normal human diploid fibroblast line are shown in Figure 3.16.

The 2N sorted 82-6 normal human fibroblasts showed an initial increase in the 4N DNA content fraction as the diploid cells resumed cycling, peaking at 10 days, before decreasing to a plateau of about 5%. As expected, these 4N cells were virtually all in the diploid G2/M phase, as tetraploid cells and pericentrin amplification were essentially non-existent in the normal fibroblast cultures (Figure 3.16 D). In the Barrett's cell cultures, the proportion of cells containing a 4N DNA content also initially increased, peaking at 10 days, before decreasing slightly to a plateau. The percentage of 4N cells in the Barrett's cultures was much higher than the fibroblast cultures (12% to 28% versus 5%). The proportion of tetraploid cells in the Barrett's esophagus cultures also gradually increased until it comprised approximately 40% of the 4N population by day 21. Unlike the 4N fraction, however, the percentage of tetraploid cells did not peak at 10 days and instead continued to rise even as the 4N fraction levelled off. This difference presumably reflects the numbers of 4N diploid G2 cells. In the Barrett's esophagus cultures, pericentrin amplification in the 4N population (defined as three or more pericentrin foci) showed a steady increase that paralleled the generation of tetraploid cells. Thus, the percentage of cells containing three or more pericentrin foci is approximately half of the percentage of tetraploids in cultures CP-94251 and KR-42421, whereas the percent of cells containing amplified pericentrin foci in CP-52731 is much lower; CP-52731 also has the lowest 4N fraction of the four cultures. The percent of pericentrin abnormalities was always less than the percent of tetraploid cells present. This is not unexpected since tetraploid cells arising from simple endoreduplication would contain only two pericentrin foci, assuming that normal centrosome duplication occurs in the diploid S phase (see Figure 3.1). The proportion of 4N cells present in a culture appears to predict the

proportion of cells displaying pericentrin amplification; a higher 4N fraction is associated with both a greater proportion of 4N cells containing amplified pericentrin foci and a greater proportion of tetraploid cells.

Elevated 4N fractions in Barrett's esophagus in vivo contain tetraploid cells

Elevated 4N fractions *in vivo* are correlated with an increased risk for subsequent development of aneuploidy (Galipeau, 1996) and cancer (Reid et al, submitted) in Barrett's esophagus. Because cells that possess a 4N DNA content can represent either diploid cells in G2 or tetraploid cells in G1, it was not clear whether the increase in the 4N fraction *in vivo* is due to a G2 arrest of diploid cells or the generation of tetraploid cells. The presence of tetraploid cells in cultured Barrett's esophagus cells derived from Barrett's esophagus patients suggests that tetraploid cells might exist *in vivo*. Tetraploidy, however, could also be an artifact caused by *in vitro* culture conditions. In order to determine whether tetraploid cells are present in Barrett's esophagus *in vivo*, we performed fluorescence in situ hybridization (FISH) analysis on cells from mucosal biopsies from patients with Barrett's esophagus. Cells derived from endoscopic biopsies of Barrett's esophagus patients were flow sorted on the basis of DNA content into 2N and 4N fractions. These were subsequently hybridized to a chromosome 18 centromeric probe in order to compare the distribution of chromosome copy number in the 2N and 4N sorted fractions. In order to avoid categorizing diploid G2 cells containing pairs of closely opposed FISH spots as tetraploid cells, only FISH signals which were clearly separated from one another were counted as individual spots. Thus, 4N cells containing two pairs of closely opposed spots were placed in the diploid (two FISH spots) category. Samples from three patients, each exhibiting different flow cytometric abnormalities, were analyzed. The FISH results and corresponding cell cycle histograms are shown in Figure 3.17. Patient 167M (Figure 3.17 A and D) did not show elevation in the 4N fraction by flow analysis (4N fraction equals 2%) and FISH analysis showed little difference in the distribution of FISH signals in 2N and 4N sorted cells. The majority of cells exhibited two FISH spots, consistent with diploidy. In contrast, patient 155P (Figure 3.17 B and E) possessed an elevated 4N fraction by flow analysis (4N fraction equals 12.5%) and FISH analysis indicated that 40% of the 4N sorted cells contained more than two FISH spots,

consistent with the existence of a tetraploid population. The cells in the samples possessing one or three FISH spots are most probably artifacts due to poor hybridization of the FISH probe or overlapping FISH spots; single spot cells are likely to be diploid and three spot cells are most likely tetraploid. Patient 117S (Figure 3.17 C and F) contained an elevated 4N fraction of 15.5% and a near-diploid aneuploidy by flow analysis. FISH of this patient's sample showed that a substantial proportion of cells in the 4N fraction contained four FISH spots (42%). This suggests that an elevation in the 4N fraction *in vivo* can be explained in part by the presence of tetraploid cells. Again, the cells possessing three FISH spots can represent artifacts of the method, or in this case, contamination of the sorted cells by aneuploid cells. The presence of cells with three hybridization signals in the 2N sorted sample is almost certainly due to the contamination of the diploid sorted cells by the near-diploid aneuploid population indicated in the flow histogram. Similarly, the presence of cells with greater than four FISH spots in the 4N sort are most likely due to contamination by aneuploid G2 cells. These findings support the hypothesis that tetraploid cells are present in elevated 4N fractions *in vivo*.

Pericentrin abnormalities are present in premalignant Barrett's esophagus and esophageal adenocarcinoma in vivo

In collaboration with Stephen Doxsey, we examined Barrett's esophagus and esophageal adenocarcinoma *in vivo* for pericentrin abnormalities. Esophageal specimens were stained for pericentrin using immunoperoxidase techniques. Pericentrin abnormalities were rarely seen in tissue samples from normal squamous esophageal epithelium and gastric mucosa. Pericentrin abnormalities were also rarely seen in Barrett's esophagus with low-grade dysplasia. However, centrosome abnormalities were always present in samples from high-grade dysplasia and invasive tumors. Structural aberrations in centrosomes including enlarged, elongated, or fragmented centrosomes were frequently seen. Some dysplastic epithelium showed decreased pericentrin staining including smaller than normal or absent pericentrin foci.

Discussion

Elevated 4N fractions in Barrett's esophagus *in vivo* are strongly predictive of subsequent development of aneuploidy (Galipeau, 1996) and cancer (Reid et al, submitted). Our work suggests that centrosome amplification in diploid G2 and tetraploid cells in the 4N fraction may contribute to the increased risk of aneuploidy and cancer in Barrett's esophagus patients. We have found that numerical and structural pericentrin abnormalities are present in cultured Barrett's esophagus cells and that cells of higher ploidy contain a greater degree of pericentrin amplification. We also observed that the 4N fraction is composed of both tetraploid G1 cells and diploid G2 cells and that both exhibit pericentrin amplification. Tetraploid cell generation occurs spontaneously *in vitro* from diploid precursor cells, but tetraploid cells appear to have a lower proliferative capacity and/or apoptose at a higher rate than diploid cells. This appears to result in a diploid-tetraploid equilibrium *in vitro* in which the generation of tetraploid cells is balanced by their demise. In general, we find that the Barrett's cell strains that develop relatively high 4N fractions from purified diploid cells also develop more FISH-confirmed tetraploid cells and contain more cells with pericentrin amplification. Similarly, we find *in vivo* that tissue samples exhibiting 4N fractions contain tetraploid cells, whereas samples containing minimal 4N fractions do not. We also observed that Barrett's esophagus tissue and esophageal adenocarcinoma *in vivo* also contain pericentrin abnormalities.

Loss of p53 function has been associated with loss of a mitotic spindle checkpoint and abnormal centrosome numbers in mice (Cross, 1995; Fukasawa, 1996). Here, we describe pericentrin amplification in precancerous Barrett's cell strains that lack p53 function. Analysis of pericentrin staining has shown amplification of centrosome numbers in the 4N and 8N fractions of unchallenged Barrett's cell strains that is similar in magnitude to those observed in $p53^{-/-}$ mouse embryonic fibroblasts challenged with nocodazole (Carissa Sanchez, unpublished results). The accumulation of cells with an elevated number of centrosomes in the Barrett's cell cultures may indicate a deficiency in either a checkpoint regulating centrosome replication (Fukasawa, 1996) or a checkpoint preventing endoreduplication after adaptation to mitotic arrest (Lanni, 1998; Khan, 1998). We examined the distribution of cells containing the expected number of centrosomes based upon the latter model. We

found that, in general, although the majority of 4N cells contained the expected number of centrosomes, there were 4N cells that contained greater than the expected number. Our cyclin B experiments showed that some amplification of centrosomes is present in the diploid G2 cells of the cultured Barrett's esophagus cells. This observation favors the mechanism of a centrosome checkpoint deficiency leading to amplified centrosomes. We further observed that although centrosome amplification occurs, there appears to be a selection for cells containing fewer centrosomes. This is most clearly illustrated by the finding that the majority of cells in the 8N sort contain fewer centrosomes than would be expected.

Diploid G2/M cells comprise, in general, only a minority of the 4N cells exhibiting pericentrin amplification. Tetraploid G1 cells make up the greater proportion of the total 4N cells exhibiting more than two pericentrin foci. Diploid G2/M cells containing amplified centrosomes must be eliminated by some mechanism since diploid G1 cells contain primarily normal centrosome numbers. We speculate that this may involve either apoptosis or conversion to tetraploidy. We found that in at least one Barrett's cell strain, that the 4N sorted culture had lower proliferative capacity and a higher apoptotic index than 2N sorted cells. This may be related to excessive centrosome numbers and illustrates the instability of the 4N population in the cultures. The spontaneous generation of tetraploid cells from diploid Barrett's cells suggests that the resumption of the cell cycle after an aborted mitosis/cytokinesis occurs. We speculate that diploid cells containing amplified centrosomes are less likely to complete mitosis in a timely manner and this may activate a mitotic spindle checkpoint (analogous to nocodazole treatment). After prolonged arrest, the mitotic diploid cell adapts and reverts to a G1 state and generation of a tetraploid cell (see Figure 3.18).

In the experiments following the generation of tetraploid from diploid cells, we observed that approximately half of the 4N cells contain three or more pericentrin foci. This may be due to centrosome amplification in the diploid cycle as described above, or it could be a result of a greater propensity of tetraploids to undergo multipolar mitosis, regardless of centrosome amplification in the diploid cycle. We would expect that tetraploid cells newly formed by aborted mitoses and/or failed

cytokinesis of a diploid cell containing two replicated centrosomes (no amplification) will only possess two centrosomes. Subsequent centrosome replication in late G1 and S phases of the tetraploid cycle would lead to formation of tetraploid cells containing four centrosomes (see Figure 3.1). It has been observed that supernumerary centrioles may cluster at one pole and allow a normal bipolar mitosis to take place in a tetraploid cells; however, unequal segregation of centrosomes in the daughter cells may occur (Ring, 1982). Bipolar mitoses that occur would generate two 4N daughters containing either two centrosomes each or one and three centrosome(s). In the latter case, we would expect that the daughter cell receiving only one centrosome would be more stable than the daughter cell receiving three centrosomes. Most importantly, cells with excessive centrosomes are more likely to undergo multipolar mitoses that can lead to the missegregation of chromosomes and generation of aneuploid cells.

An unstable tetraploid intermediate has been proposed as a precursor to the development of aneuploidy in human cancers (Shackney, 1989). Examination of tumor tissue and cell lines has suggested that doubling of the chromosome content followed by loss of chromosomes is a common event leading to aneuploidy (Burholt, 1989; Shackney, 1995a; Shackney, 1995b; Jin, 1995). Barrett's esophagus is the clearest example of a premalignant human condition in which increased 4N fractions are known to be a strong indicator of future aneuploidy and cancer development. Our data suggests that the elevated risk for development of aneuploidy and cancer that is associated with elevated 4N fractions is due to the presence of tetraploid cells with abnormal numbers of centrosomes. We show that tetraploid cells do exist in Barrett's esophagus *in vivo*, and appear to be correlated with the degree of flow cytometric 4N abnormality. Centrosome abnormalities are present in dysplastic areas of Barrett's esophagus. We speculate that centrosome amplification and tetraploid cell generation contribute to the cancer risk in Barrett's esophagus patients exhibiting 4N flow cytometric abnormalities.

Methodology

Cell culture maintenance

Barrett's esophagus cell strains (CP-18821; CP-52731; CP-94251; KR-42421) were maintained in MCDB 153 containing growth supplements as previously described (Chapter 1; Palanca-Wessels, 1998). Normal human diploid fibroblasts (82-6; LG EF) were grown in DMEM containing 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin.

Flow cytometry

DNA content flow cytometric sorting was used to purify different ploidy populations from both *in vivo* and *in vitro* samples using a Coulter Elite cell sorter (Miami, FL). For *in vivo* samples, biopsies were processed before sorting as described previously (Neshat, 1994a; Blount, 1994). For viable sorting of cultured cells, cells were incubated in growth medium containing Hoescht 33342 (Calbiochem, La Jolla, CA) at 10 μM concentration for 30 minutes at 37°C. Cells were trypsinized after incubation, resuspended in new growth media containing dye, and kept covered at room temperature until sorting. For DAPI staining, cultured cells were trypsinized and resuspended in 10 μg/ml DAPI in NST buffer [146 mM NaCl, 10 mM Tris base, 0.1% NP40 (Sigma), 2 mM Ca, 20 mM Mg, and 0.05% BSA, pH 7.4] before analysis or sorting. The Multiplus software program (Phoenix Flow Systems, San Diego, CA) developed by Dr. Peter S. Rabinovitch was used for cell cycle analysis.

Immunocytochemistry

Pericentrin staining was performed as follows. Viably sorted cells were allowed to adhere to Lab-Tek chambered glass slides (Nalge Nunc International, Naperville, IL) coated with human fibronectin (Sigma, St. Louis, MO) for three to five hours post-sort. Slides were rinsed in 1X Hanks buffered saline solution (Gibco, Grand Island, NY) then covered with PIPES buffer (80 mM PIPES, 5 mM EGTA, 1 mM $MgCl_2$) for 2 minutes followed by a 5 minute fixation in cold methanol at -20°C. The slides were incubated in PBA buffer (1X phosphate-buffered saline, 1% bovine serum albumin, 0.1% Triton-X, 10% normal goat serum) for at least 15 minutes. The slides were then covered with affinity

purified anti-pericentrin antibody (M1-100) (a gift from Stephen J. Doxsey) for 1 hour, rinsed in PBA buffer, then incubated with a fluorescein isothiocyanate (FITC)-labelled secondary goat anti-rabbit antibody (Cappel, Durham, North Carolina) for 30 minutes. Slides were washed in PBA buffer and counterstained with DAPI and mounted with DABCO (Sigma). Counts were performed using a fluorescence microscope and at least 200 cells were counted for each sort when possible. For simultaneous cylin b staining, cells were incubated with mouse anti-human cyclin b antibody GNS-1 (Pharmingen, San Diego,CA) diluted 1:1000 in PBA buffer overnight prior to staining with pericentrin antibody. Cy3-labelled secondary goat anti-mouse antibody (Caltag, Burlingame, CA) was added to the FITC labelled secondary goat anti-rabbit antibody during the incubation step following staining with the anti-pericentrin antibody.

Fluorescence in situ hybridization

In situ hybridization was performed as follows. Viably sorted cultured cells were allowed to adhere to slides (three to five hours post-sort) before fixation. Cells derived from endoscopic biopsies were dropped directly on Cell-Tak™ (Collaborative, Bedford, MA) coated slides and allowed to air-dry prior to fixation. Slides were fixed with 3:1 methanol to acetic acid for 10 minutes then allowed to dry overnight. Slides were incubated in pre-rinse solution (5X SSC, 0.5% Tween 20) at 65°C for 5 minutes followed by a series of room temperature ethanol washes (70%, 85%, 100%) for 2 minutes each. Denaturation was performed in a formamide (United States Biochemical, Cleveland, OH) and SSC (Gibco, Grand Island, NY) solution (70% formamide, 30% SSC) for 3 minutes at 72°C. Slides were put through a series of -20°C ethanol washes (70%, 85%, 100%) for 2 minutes each, then allowed to dry. Directly-conjugated fluorescein isothiocyanate (FITC) and/or Texas Red (TR) alpha-satellite centromeric probes (Oncor, Gaithersburg, MD) were applied to the slides and allowed to hybridize for at least 12 hours at 37°C in a humidified chamber. After hybridization, slides were incubated in 0.25X SSC at 72°C for 5 minutes to remove excess probe, then washed three times (0.25X SSC, 0.03% Tween 20, pH 5.3) before mounting in antifade containing DAPI (Oncor).

Table 3.1 Growth and apoptosis in diploid and tetraploid Barrett's esophagus cells in vitro
Cells were sorted into 2N and 4N fractions. 2N and 4N sorted cells were recultured for 2 weeks. 2N cultures were assayed for growth and apoptosis. 4N cells were re-sorted into 2N and 4N fractions (second sorts) and cultured for another 2 weeks before being assayed for growth and apoptosis. S.E.M.; standard error of the mean.

Cell Strain	*Sorted fraction*	*Growth since sort (PDLs) (average ±S.E.M.)*	*Apoptotic cell (average ±S.E.M.)*
KR-42421	cultured 2N (first sort)	2.5 ± 0.2	1.1% ± 0.0%
	cultured 2N (second sort)	3.7 ± 0.2	3.0% ± 0.1%
	cultured 4N (second sort)	1.1 ± 0.2	7.1% ± 0.5%
CP-94251	cultured 2N (first sort)	4.7 ± 0.1	2.2% ± 0.2%
	cultured 2N (second sort)	4.1 ± 0.1	0.5% ± 0.1%
	cultured 4N (second sort)	4.2 ± 0.1	0.9% ± 0.0%

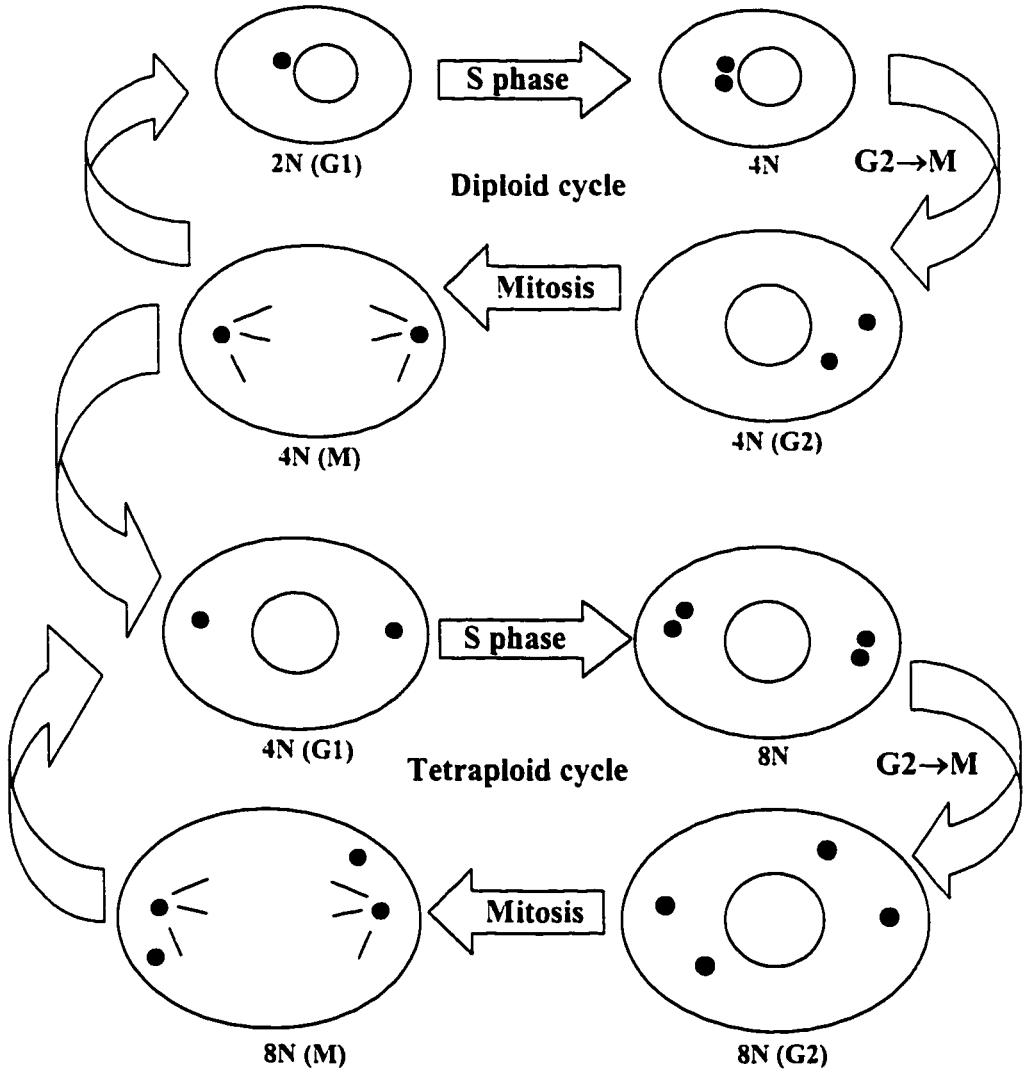


Figure 3.1 Schematic representation of centrosome replication in diploid and tetraploid cell cycles. The model assumes that normal centrsome replication and conventional endoreduplication occurs. Diploid G1(2N) cells contain one centrosome (filled circle) which is duplicated during S phase. Duplicated centrsomes in diploid G2/M (4N) cells begin to separate in preparation for mitosis. The centrosomes form the spindle poles in a bipolar mitosis. If normal mitosis and cytokinesis occurs, two daughter cells are generated,each containing a single centrosome. If mitosis is aborted and cytokinesis does not occur, the 4N cell reverts to a tetraploid G1 state with a 4N DNA content and containing two centrosomes. The tetraploid cell can enter S phase and centrosome replication occurs giving rise to a tetraploid G2/M cell (8N) containing four centrosomes. The four centrosomes separate in preparation for tetraploid mitosis (M). Depending upon orientation and spindle pole assembly, a normal bipolar mitosis may occur where only two centrosomes organize spindle fibers (shown) and normal mitosis occurs. Alternatively, a multipolar spindle may form resulting in a multipolar mitosis. In either case, segregation of centrosomes may result in an uneven distribution of centrosomes to the daughter cells from the tetraploid mitosis.

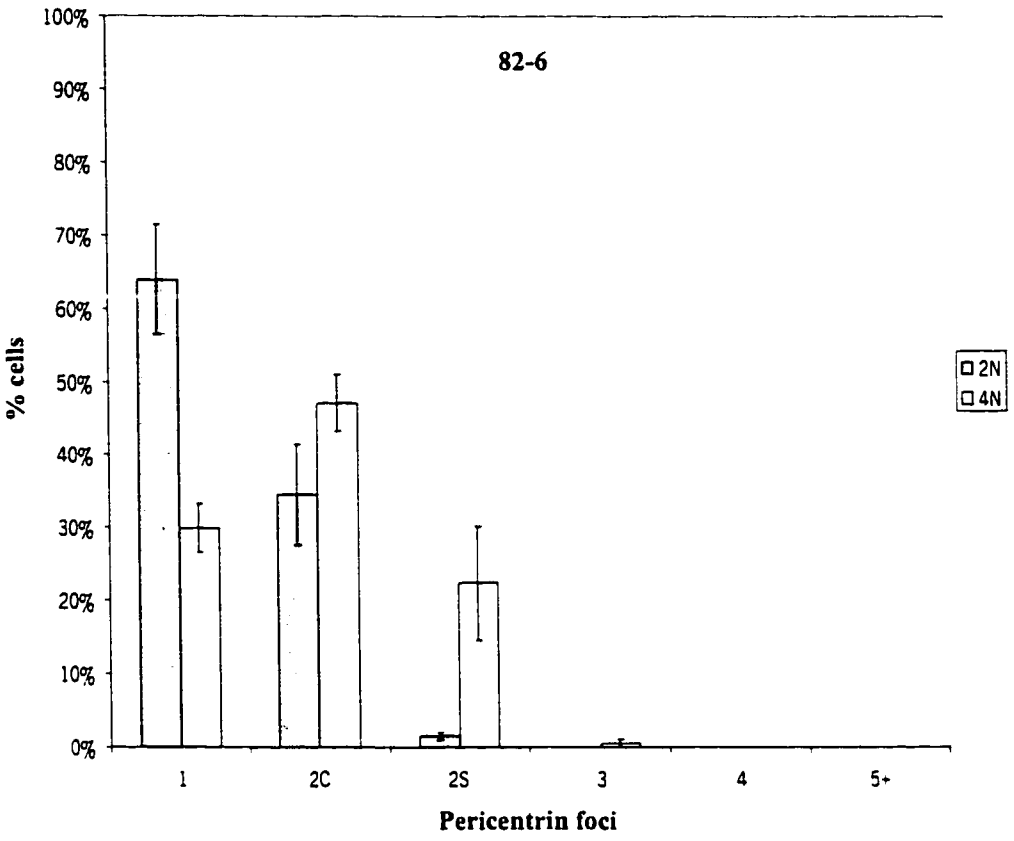


Figure 3.2 Pericentrin foci distribution in normal human fibroblasts. Normal human diploid fibroblasts (82-6) were sorted by ploidy into separate 2N (light gray bar) and 4N (white bar) fractions. After allowing sorted cells to adhere to slides, cells were fixed and stained for pericentrin (about 5 hours post-sorting). The number of pericentrin foci was recorded and the distribution of cells possessing increasing number of pericentrin foci is shown above. Cells with two foci were scored as either possessing two closely spaced spots (2C) or two separated spots (2C). Cells containing five or more foci are grouped in the 5+ category. The error bars represent the standard error of the mean from two separate experiments.

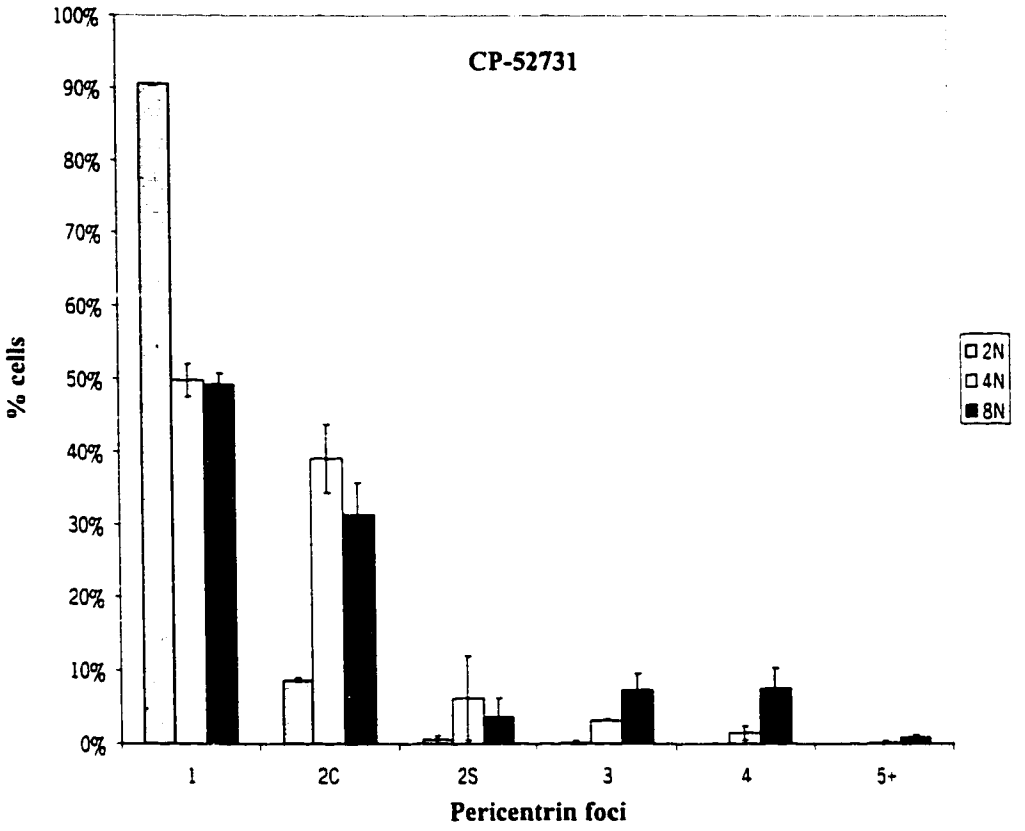


Figure 3.3 Pericentrin foci distribution in Barrett's esophagus cell strain CP-52731. Barrett's cells were sorted by ploidy into separate 2N (light gray bar), 4N (white bar), and 8N (dark gray bar) fractions. After allowing sorted cells to adhere to slides, cells were fixed and stained for pericentrin (about 5 hours post-sorting). The number of pericentrin foci was recorded and the distribution of cells possessing increasing number of pericentrin foci is shown above. Cells with two foci were scored as either possessing two closely spaced spots (2C) or two separated spots (2C). Cells containing five or more foci are grouped in the 5+ category. The error bars represent the standard error of the mean from two separate experiments.

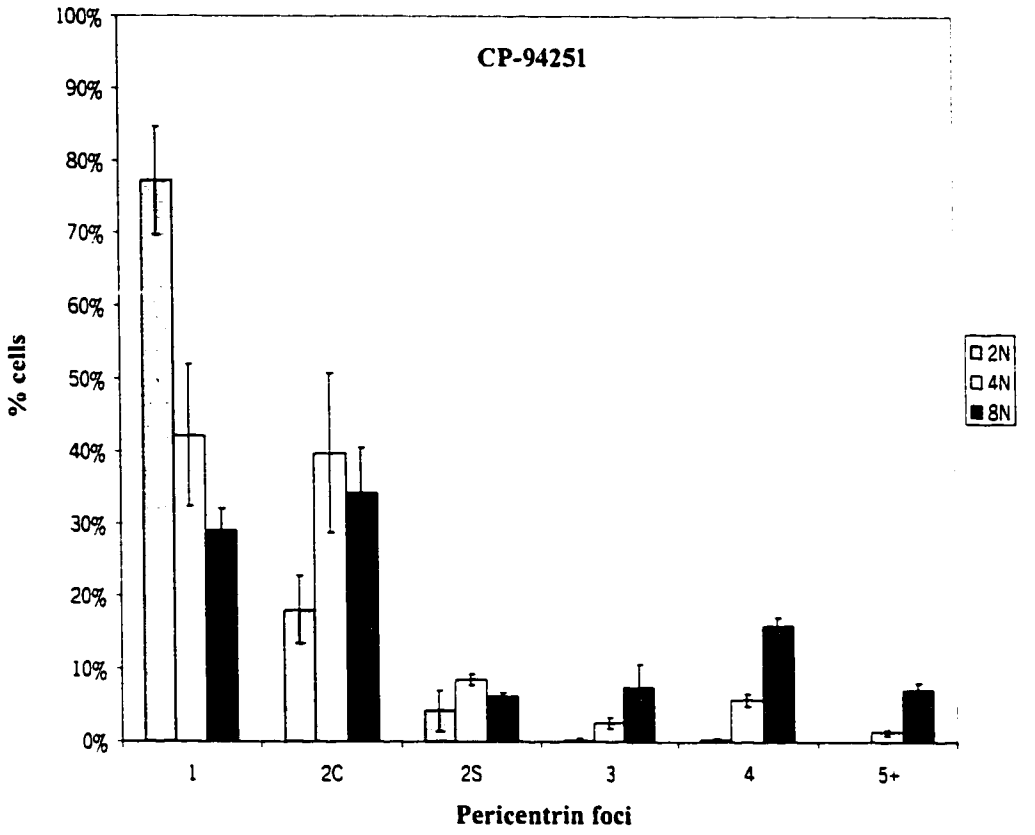


Figure 3.4 Pericentrin foci distribution in Barrett's esophagus cell strain CP-94251. Barrett's cells were sorted by ploidy into separate 2N (light gray bar), 4N (white bar), and 8N (dark gray bar) fractions. After allowing sorted cells to adhere to slides, cells were fixed and stained for pericentrin (about 5 hours post-sorting). The number of pericentrin foci was recorded and the distribution of cells possessing increasing number of pericentrin foci is shown above. Cells with two foci were scored as either possessing two closely spaced spots (2C) or two separated spots (2C). Cells containing five or more foci are grouped in the 5+ category. The error bars represent the standard error of the mean from two separate experiments.

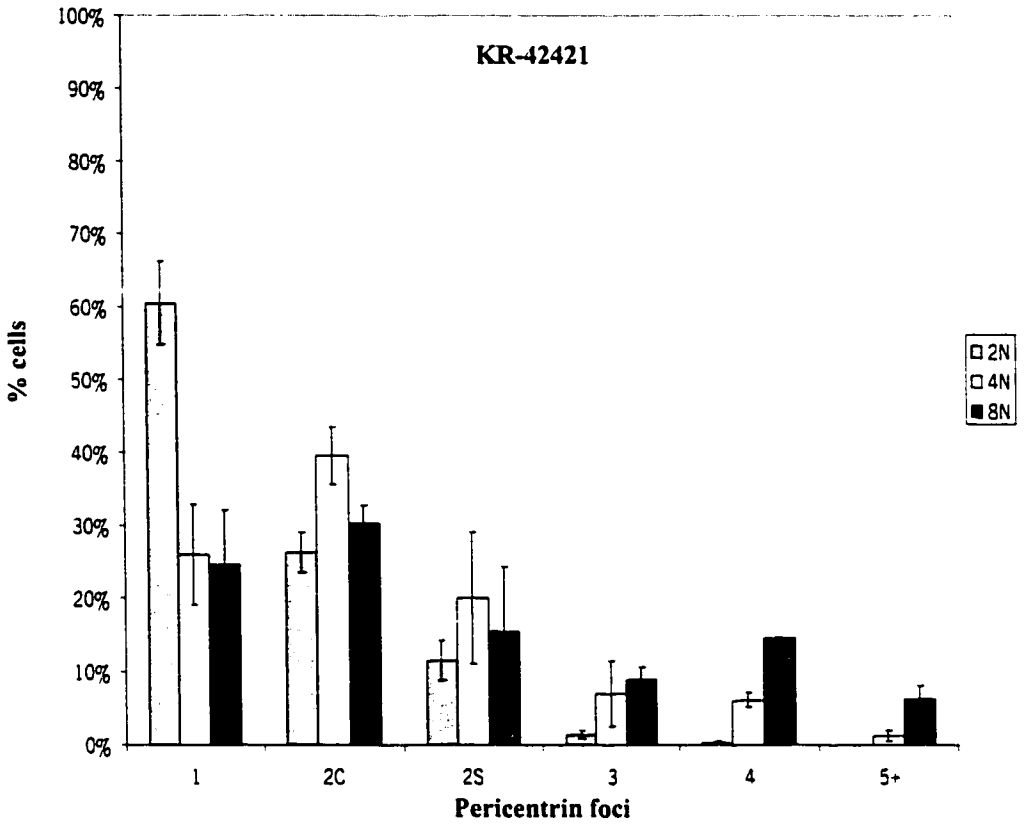


Figure 3.5 Pericentrin foci distribution in Barrett's esophagus cell strain KR-42421. Barrett's cells were sorted by ploidy into separate 2N (light gray bar), 4N (white bar), and 8N (dark gray bar) fractions. After allowing sorted cells to adhere to slides, cells were fixed and stained for pericentrin (about 5 hours post-sorting). The number of pericentrin foci was recorded and the distribution of cells possessing increasing number of pericentrin foci is shown above. Cells with two foci were scored as either possessing two closely spaced spots (2C) or two separated spots (2C). Cells containing five or more foci are grouped in the 5+ category. The error bars represent the standard error of the mean from two separate experiments.

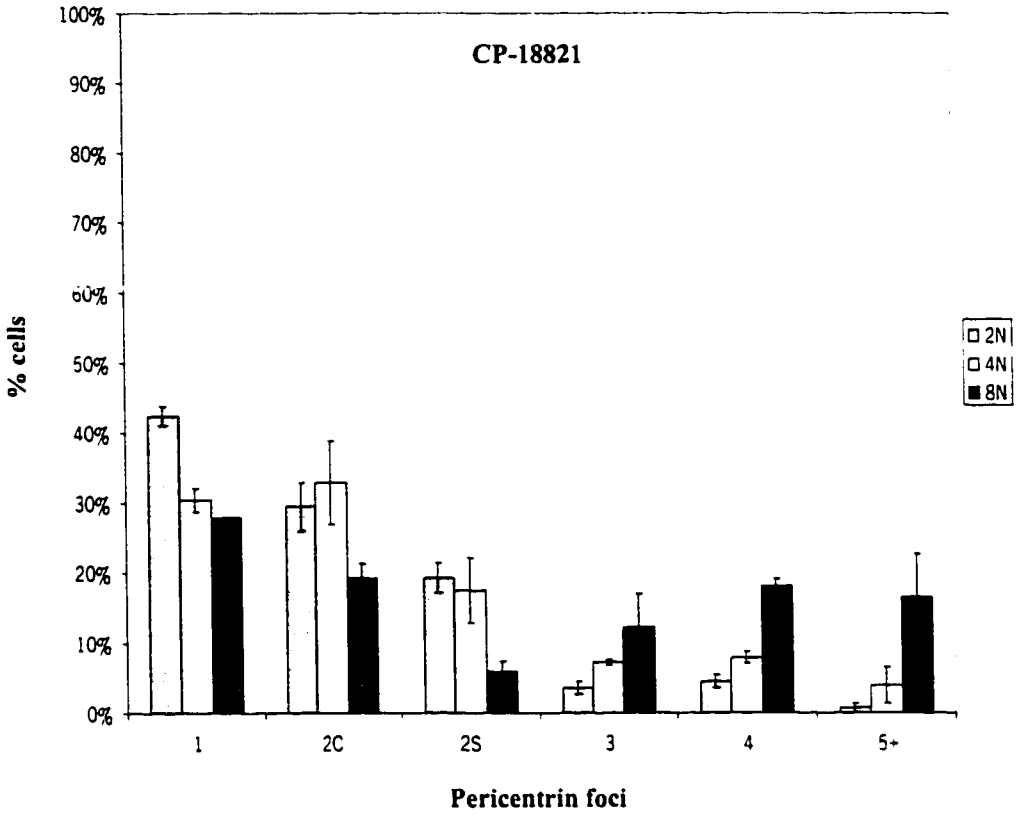


Figure 3.6 Pericentrin foci distribution in Barrett's esophagus cell strain CP-18821. Barrett's cells were sorted by ploidy into separate 2N (light gray bar), 4N (white bar), and 8N (dark gray bar) fractions. After allowing sorted cells to adhere to slides, cells were fixed and stained for pericentrin (about 5 hours post-sorting). The number of pericentrin foci was recorded and the distribution of cells possessing increasing number of pericentrin foci is shown above. Cells with two foci were scored as either possessing two closely spaced spots (2C) or two separated spots (2C). Cells containing five or more foci are grouped in the 5+ category. The error bars represent the standard error of the mean from two separate experiments.

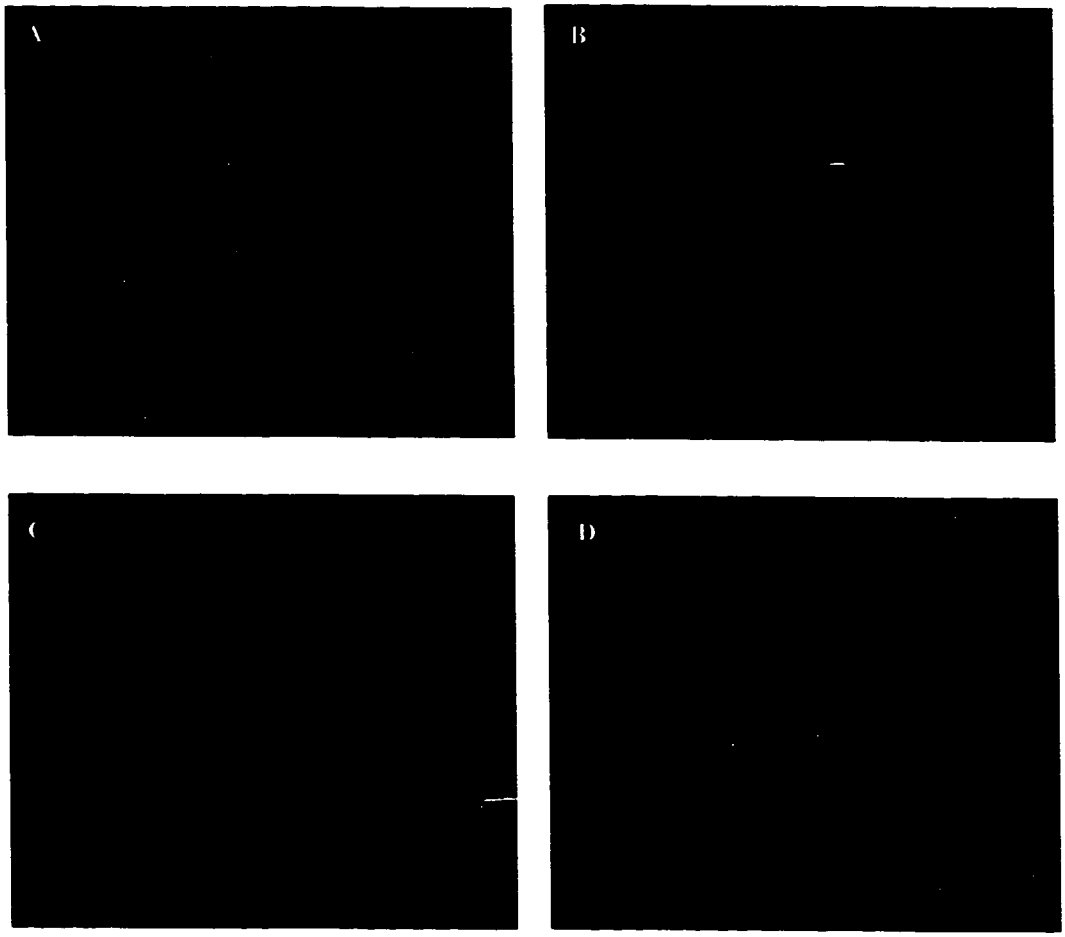


Figure 3.7 Examples of pericentrin staining. Shown are cells stained with pericentrin antibody (green) and DAPI (a blue nuclear stain). Normal fibroblasts exhibiting *A*, single pericentrin foci in the 2N sort and *B*, two separated foci in the 4N sort. Barrett's cells containing *C*, four foci (two closely positioned and two separated) in the 4N sort and *D*, a large cluster of foci in the 8N sort.

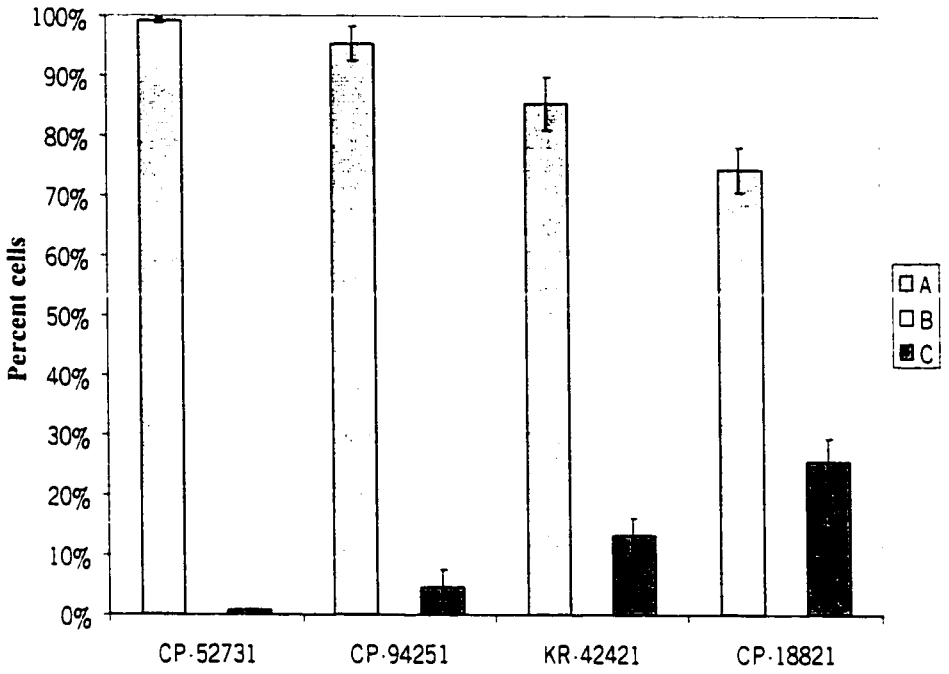


Figure 3.8 Pericentrin foci distribution of 2N sorted Barrett's esophagus cells based on endoreduplication model. The percentage of cells containing; A, the expected number of foci (one or two closely spaced); B, less than the expected number of foci (zero); C, greater than the expected number of foci (two separated or greater than three).

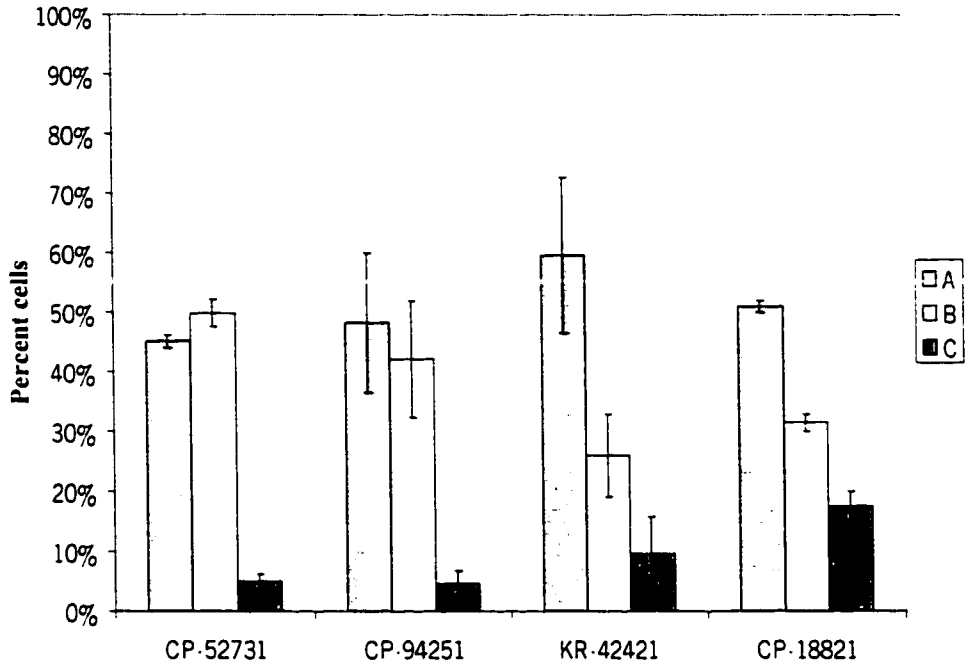


Figure 3.9 Pericentrin foci distribution of 4N sorted Barrett's esophagus cells based on endoreduplication model. The percentage of cells containing; A, the expected number of foci (two closely spaced or two separated); B, less than the expected number of foci (one); C, greater than the expected number of foci (three or more).

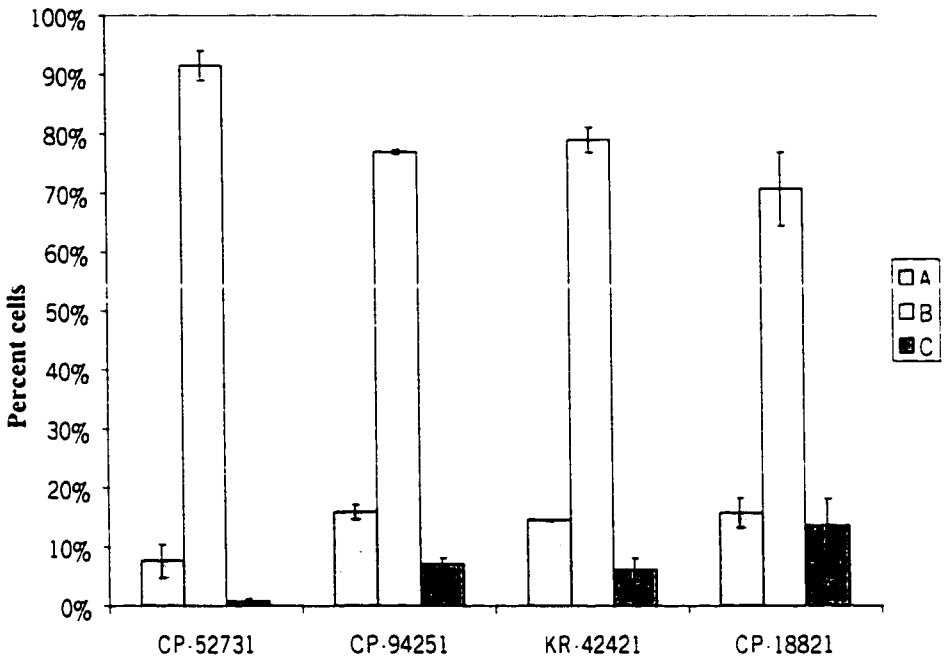


Figure 3.10 Pericentrin foci distribution of 8N sorted Barrett's esophagus cells based on endoreduplication model. The percentage of cells containing; A, the expected number of foci (four); B, less than the expected number of foci (three or less); C, greater than the expected number of foci (five or more).

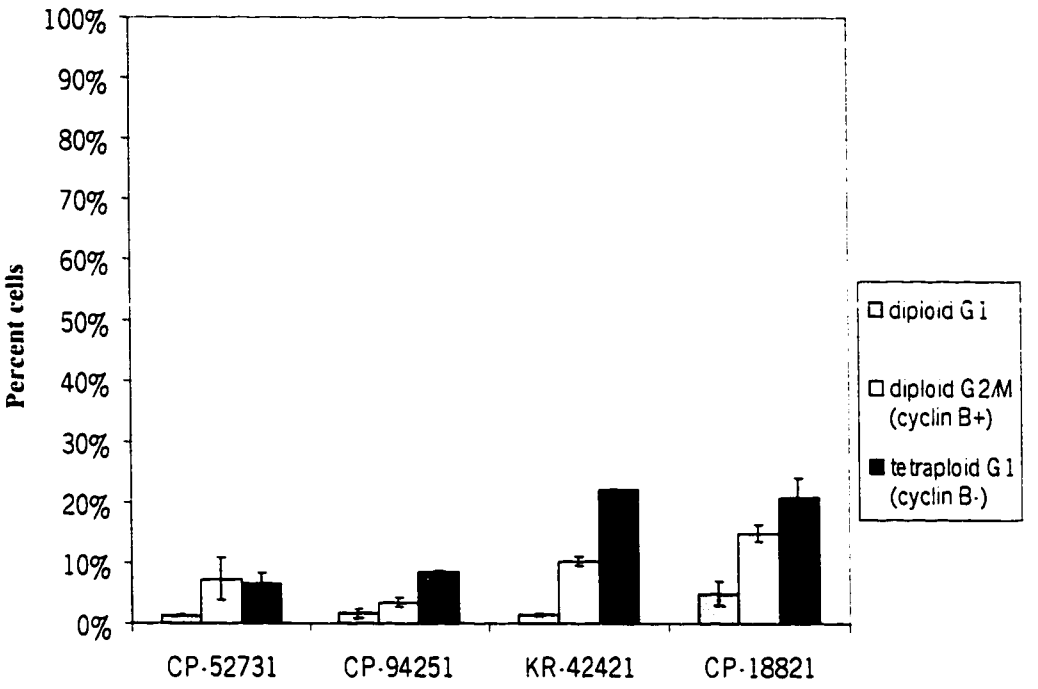


Figure 3.11 Pericentrin amplification in diploid G1 (light gray bar), diploid G2/M (white bar), and tetraploid G1 (dark gray bar) Barrett's cells. 2N and 4N sorted cells from Barrett's esophagus cell strains were allowed to adhere in the presence of nocodazole, then immunostained for both pericentrin and cyclin b expression. 4N sorted cells containing high cyclin B were identified as diploid cells in the late G2/M phase of the cell cycle while 4N sorted cells containing low cyclin B were identified as tetraploid G1 cells. The percent of cells in each category (diploid G1, diploid G2/M, and tetraploid G1) containing three or more pericentrin foci is shown above. The error bars represent the standard error of the mean between two or more experiments.

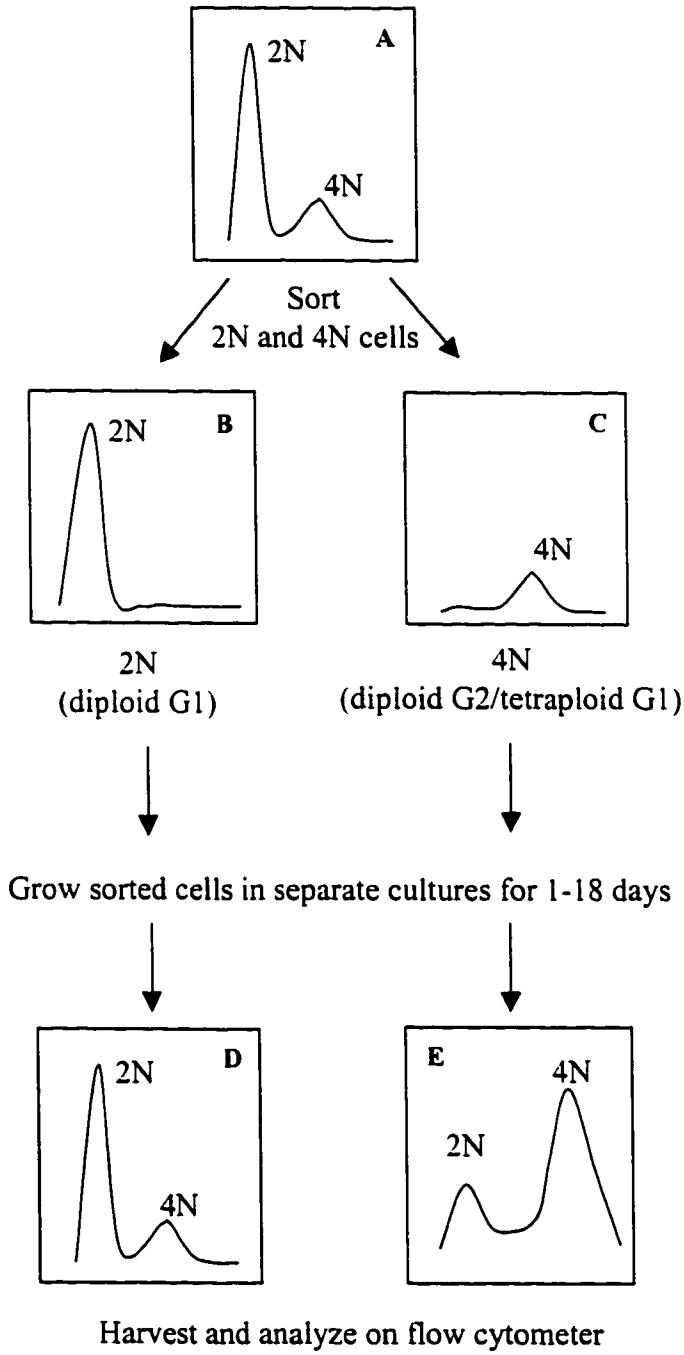


Figure 3.12 Schematic diagram of cell sorting experiments examining diploid-tetraploid equilibrium. Asynchronous cultures containing 2N and 4N populations (*Panel A*) were sorted by ploidy. The 2N (*Panel B*) and 4N *(Panel C*) cells were returned to culture and grown separately. Cells were harvested at various times after sorting and re-analyzed on the flow cytometer (*Panels D and E*). 2N sorted cells lose synchrony and as well as generate tetraploid cells. 4N sorted cells resolve into diploid and tetraploid populations.

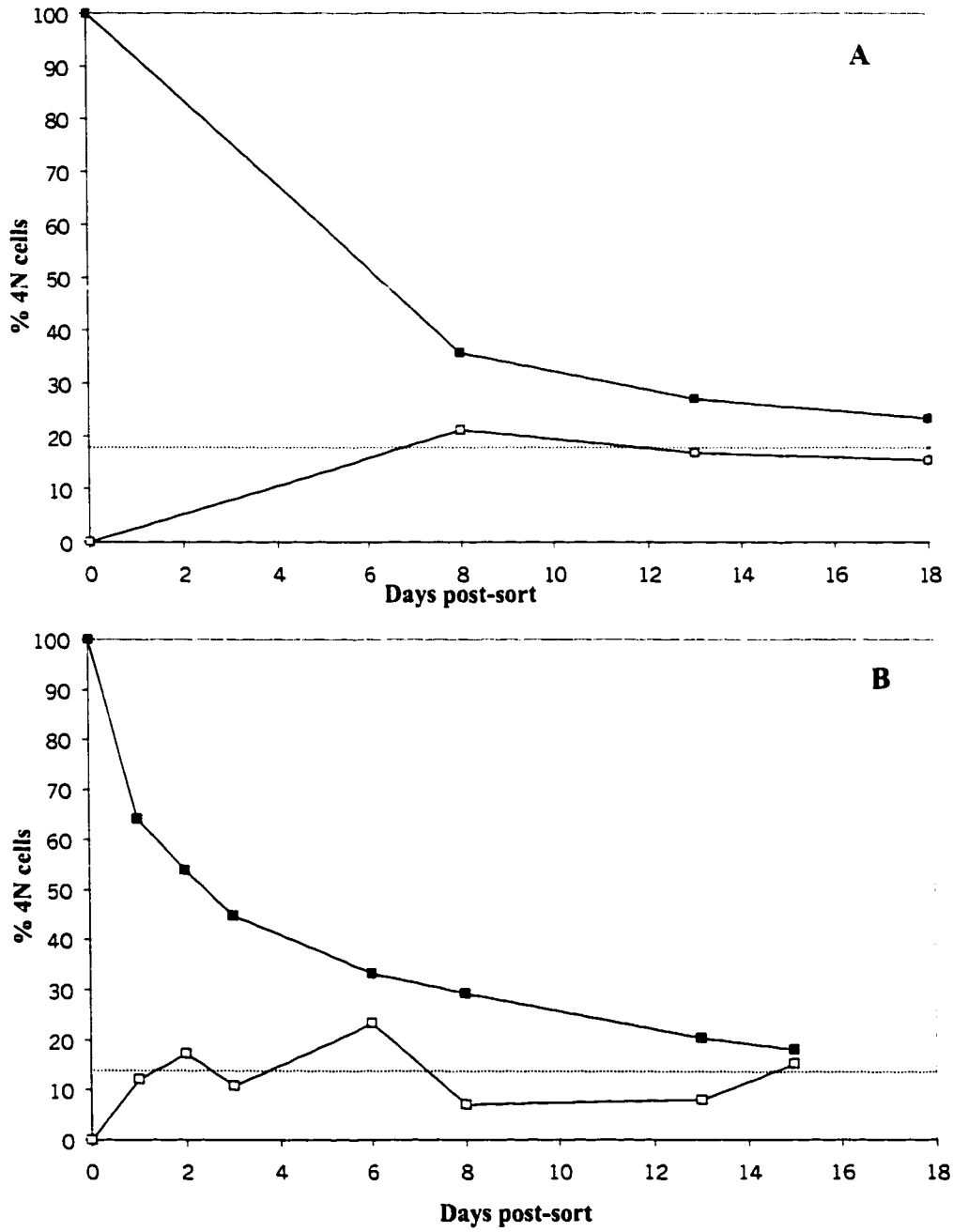


Figure 3.13 Diploid-tetraploid equilibrium exists in Barrett's esophagus cultures. Asynchronous cultured Barrett's cells (CP-52731) were sorted on two separate occasions (*A* and *B*) into 2N and 4N fractions. These were kept separate and returned to culture then re-analyzed on the flow cytometer at various times post-sorting. The percent of 4N cells found in the 2N (open squares) and 4N (solid squares) cultures are shown above. The dashed line indicates the percent of 4N cells existing in the asynchronous cultures before the initial sort.

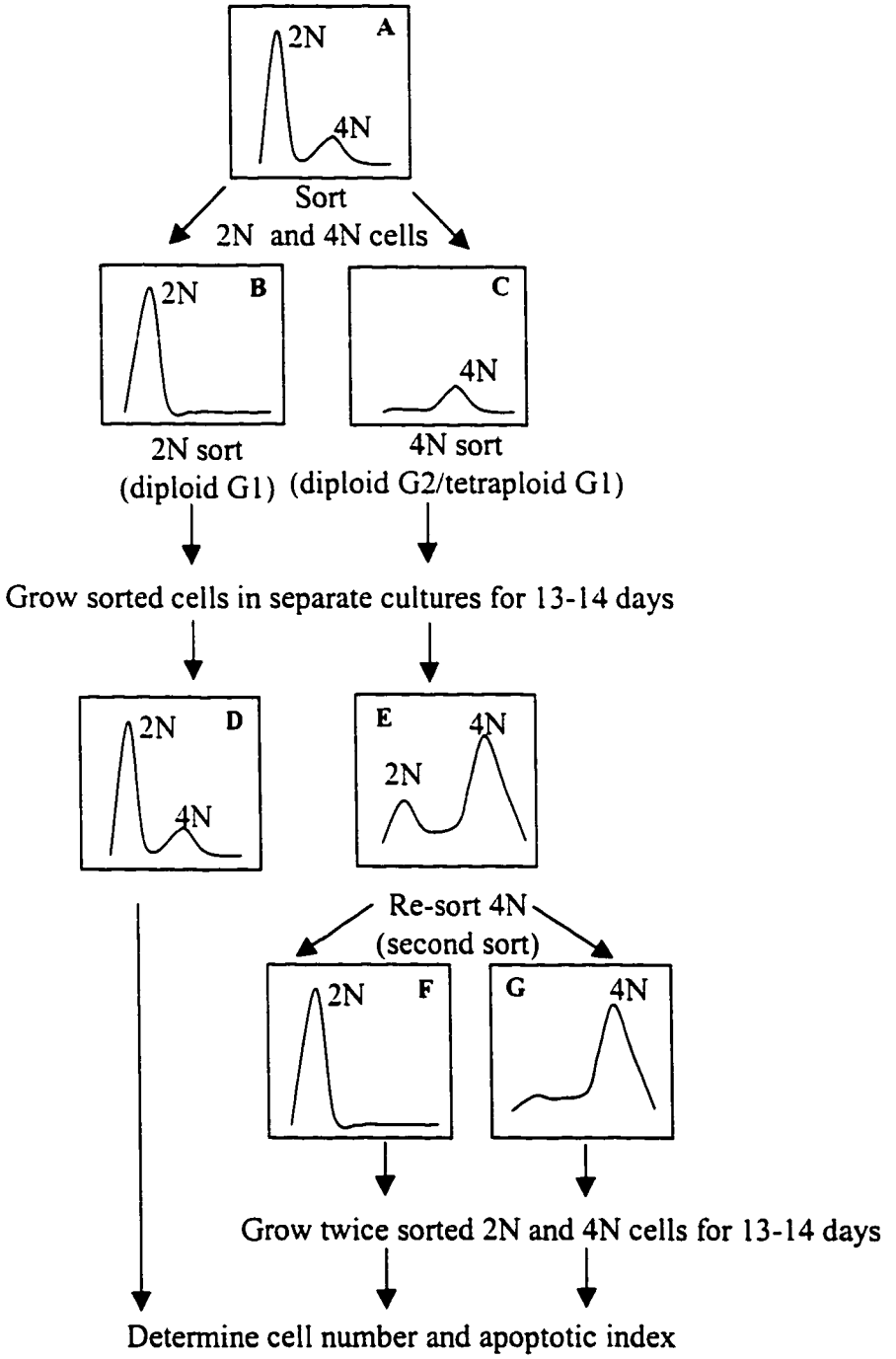


Figure 3.14 Schematic diagram of cell sorting experiments examining relative growth and apoptosis in the 2N versus 4N fraction. Asynchronous cultures containing 2N and 4N populations (*Panel A*) were sorted by ploidy. The 2N (*Panel B*) and 4N *(Panel C*) cells were returned to culture and grown separately. After 13-14 days of growth, the 2N culture (*Panel D*) was harvested, counted, and assayed for apoptosis. The 4N culture (*Panel E*) was re-sorted into 2N (*Panel F*) and 4N cells (*Panel G*) and grown for 13-14 days. Cells were then harvested, counted, and assayed for apoptosis.

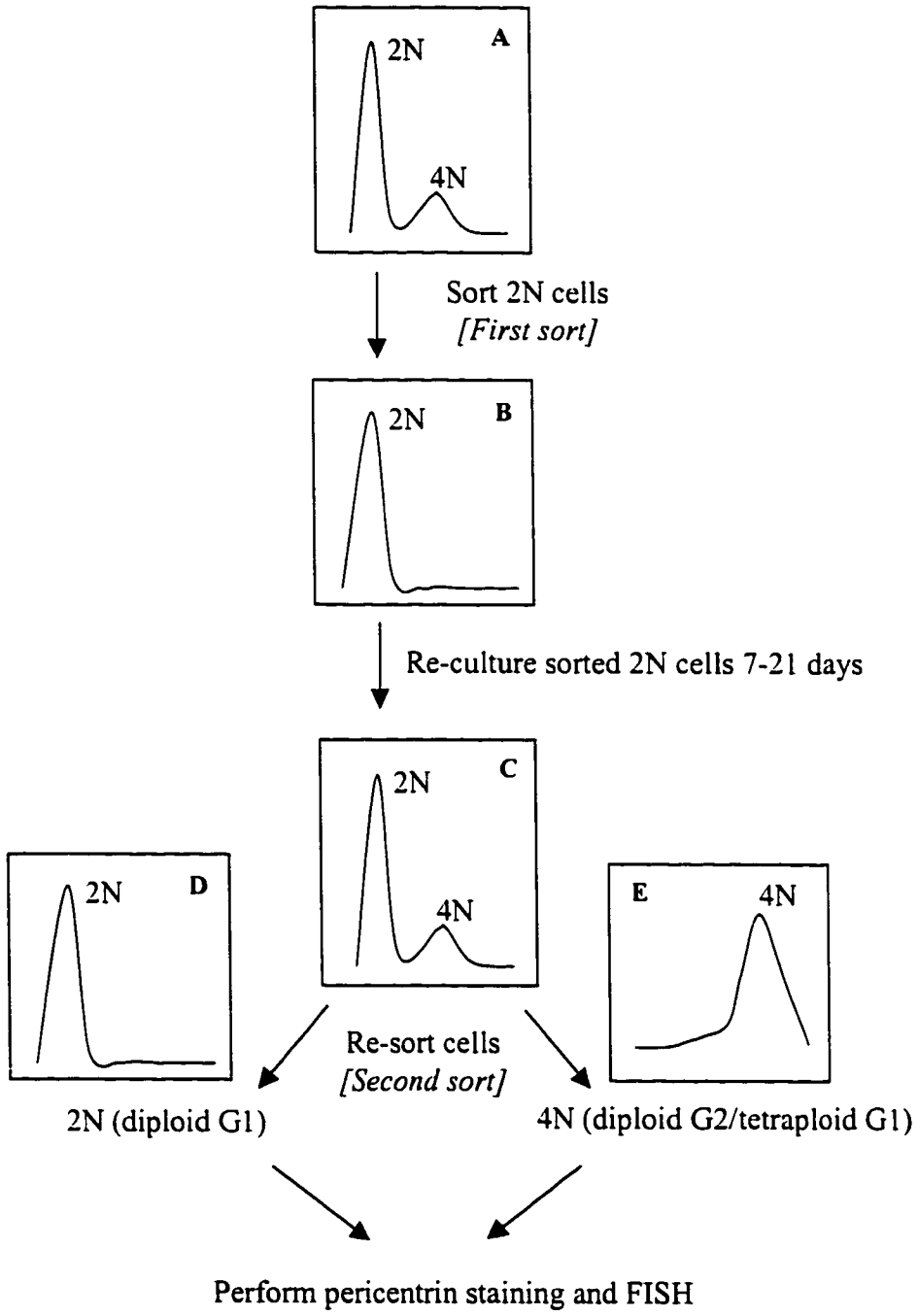


Figure 3.15 Schematic diagram of cell sorting experiments. Asynchronous cultures containing 2N and 4N populations (*Panel A*) were sorted by ploidy and the 2N cells returned to culture (*Panel B*). The sorted 2N cells resumed growth and regenerated a distinct 4N population due to loss of synchrony and generation of tetraploid cells (*Panel C*). At various timepoints, the cells were re-sorted into 2N (*Panel D*) and 4N (*Panel E*) cells for further pericentrin and FISH analyses.

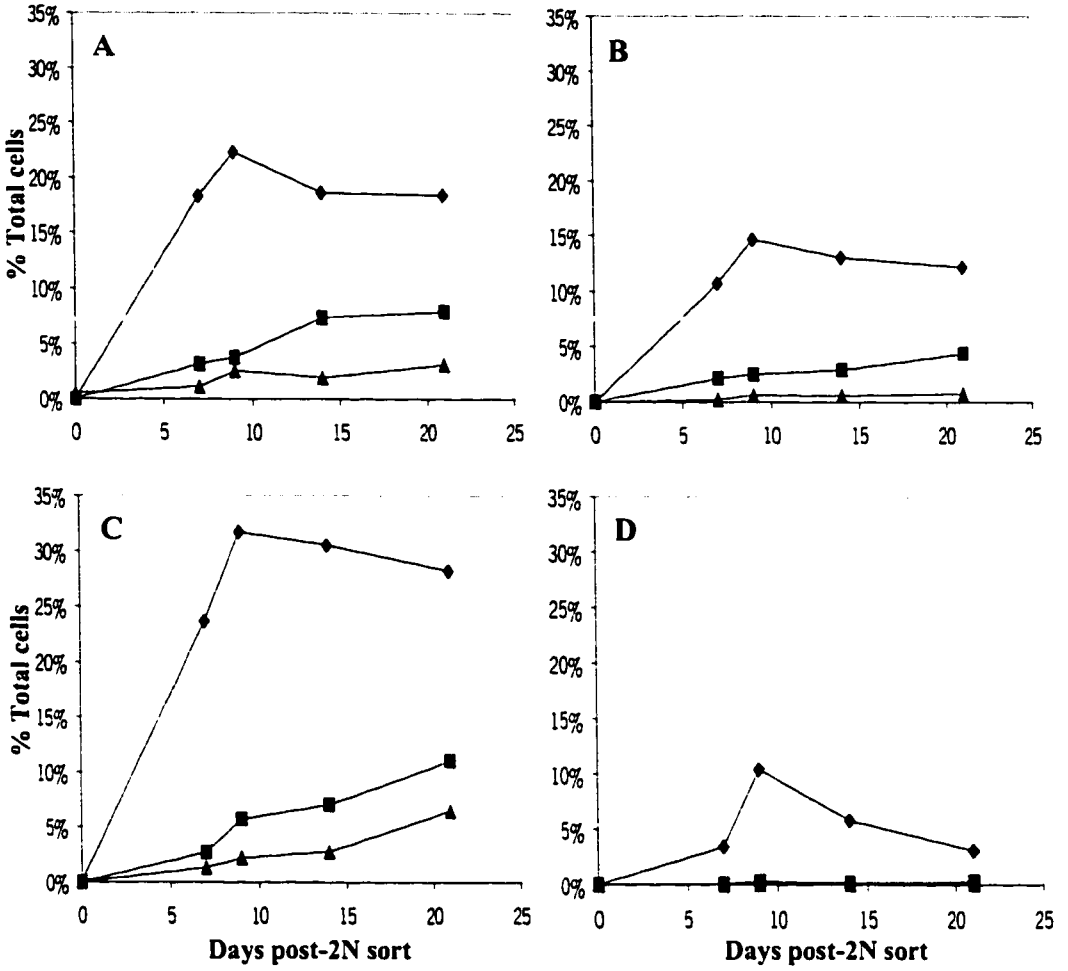


Figure 3.16 Tetraploidization and pericentrin abnormalities arise spontaneously in Barrett's esophagus cultured cells but not in normal human fibroblasts. 2N cells were sorted and analyzed for both pericentrin and ploidy in three Barrett's esophagus cell strains and one normal human fibroblast line at various timepoints: *A*, CP-94251; *B*, CP-52731; *C*, KR-42421; *D*, 82-6. At various times after the initial sorting, sorted cultures were analyzed for 4N fraction by flow cytometry (◆), tetraploidy by FISH (■), and pericentrin amplification defined as three or more pericentrin foci (▲). The zero timepoint represents the data from the original 2N sorted population after the initial sorting.

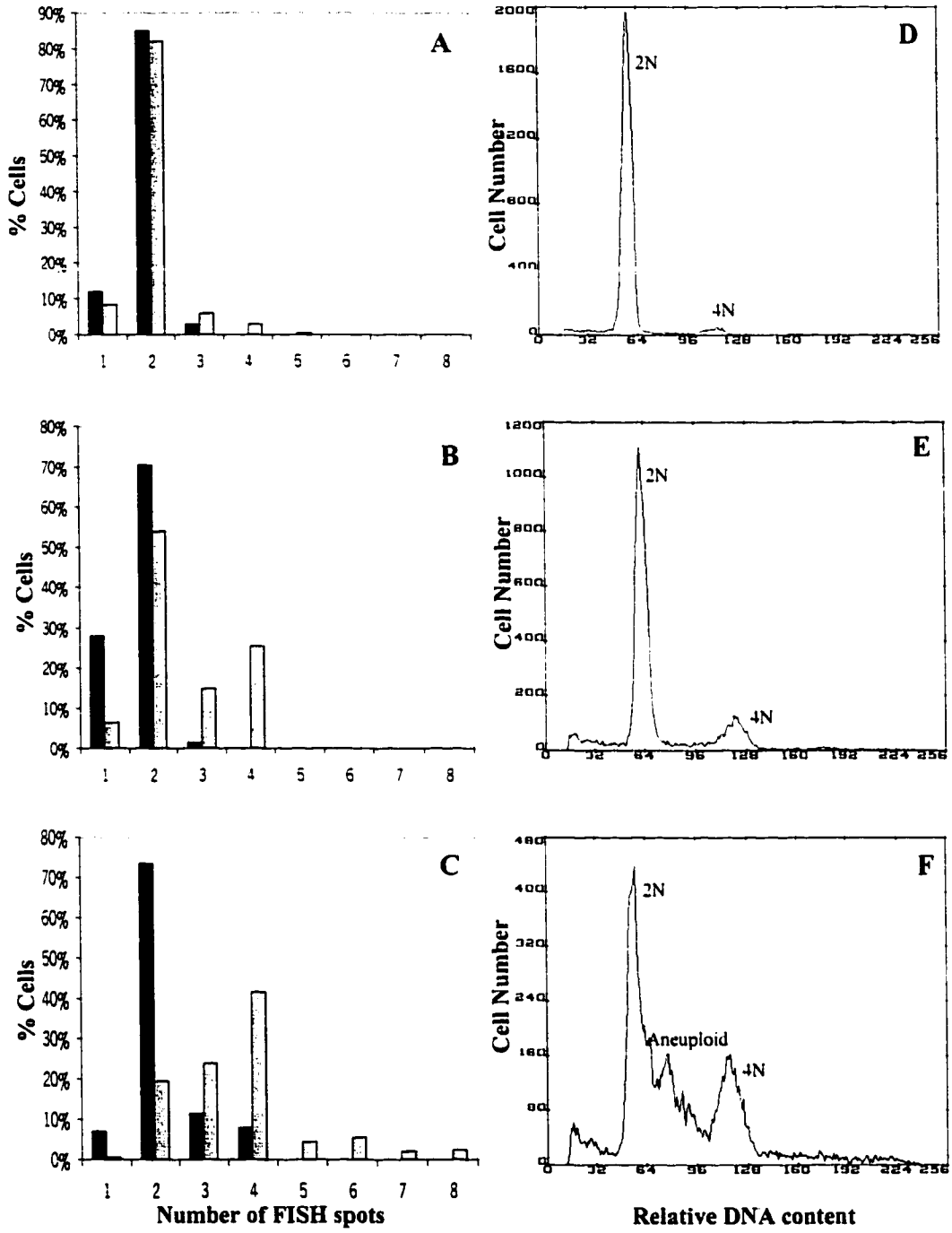


Figure 3.17 FISH analysis of sorted Barrett's patient samples reveals the presence of tetraploid cells in elevated 4N fractions. FISH was performed using a centromeric probe to chromosome 18 on sorted 2N (black bar) and 4N (gray bar) samples from three patients with different flow cytometric 4N abnormalities. The distribution of FISH spots and the accompanying DNA histogram are shown: *A* and *D*, normal 4N (patient 167M); *B* and *E*, elevated 4N (patient 155P); *C* and *F*, elevated 4N and near-diploid aneuploidy (patient 117S).

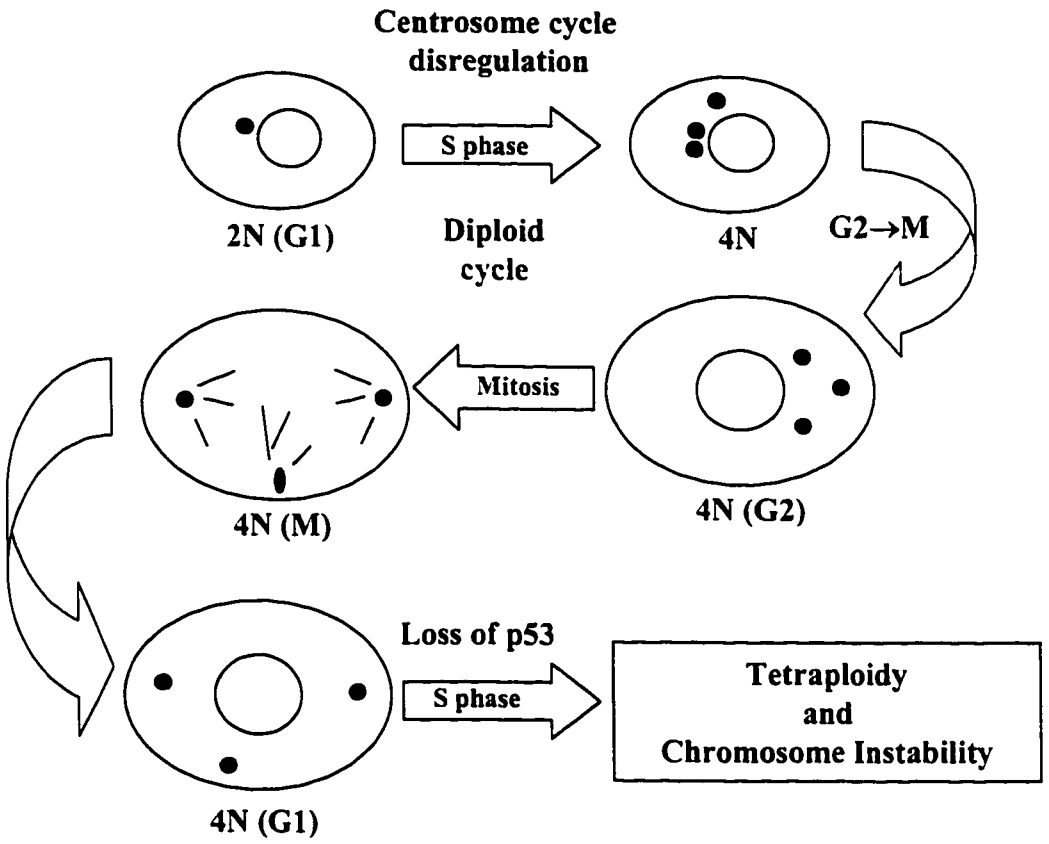


Figure 3.18 Proposed model for the role of centrosome abnormalities in tetraploidization and chromosome instability. A diploid G1 cell containing a single centrosome (filled circle) enters S phase during which time centrosome duplication occurs. Centrosome cycle disregulation can occur in the cell leading to the formation of more than two centrosomes (amplified centrosomes). Diploid G2 cells with more than two centrosomes can undergo normal bipolar mitosis if the centrosomes align on a bipolar axis. If centrosome disregulation leads to large numbers of centrosomes (four or more), this can result in the generation of a diploid cell G1 with amplified centrosomes as well. Diploid G2 cells with more than two centrosomes may have problems organizing a bipolar spindle and may attempt to undergo a multipolar mitosis. This may lead to a prolonged mitotic arrest leading to adaptation and reversion to a G1 tetraploid state similar to what occurs with prolonged mitotic arrest during continuous exposure to nocodazole. Loss of p53 allows the cells to re-enter the cell cycle and form cycling tetraploid populations. Tetraploid cells containing excessive numbers of centrsomes are chromosomally unstable due to increased probability of multipolar mitosis.

Chapter 4: Immortalization of Barrett's Epithelium by Human Telomerase Catalytic Subunit

Introduction

Progressive shortening of telomeres during the course of ongoing cell division occurs as a consequence of the inability of DNA polymerase to fully replicate the ends of chromosomes (the end-replication problem) (Olovnikov, 1973). With each successive mitosis in the absence of telomerase activity, chromosome ends become shorter and shorter (Harley, 1990). It has been suggested that this represents a biological clock which monitors the number of mitotic divisions a particular cell has undergone. The finite number of cell divisions observed in cells *in vitro*, the Hayflick limit, has been hypothesized to be due to the gradual reduction in telomere length (Hayflick, 1965). Replicative senescence occurs when telomere length decreases to a certain point, at which time a p53-dependent pathway is activated causing a G1 arrest at senescence (Campisi, 1997). Cells in which p53 is inactivated, for example by simian virus 40 (SV40) large T-antigen or human papilloma virus E6/E7 proteins, can bypass senescence (mortality stage 1 or M1) and continue to divide until they reach crisis, at which point the telomere ends have shortened to such a critical point that chromosomes become unstable and massive cell death occurs (mortality stage 2 or M2). Spontaneously immortalized cells survive crisis, typically having lengthened telomeres and containing telomerase activity, although alternate pathways of telomere maintenance may be present (Bryan, 1997; Bryan, 1995; Rogan, 1995; Counter, 1992).

Telomerase is a ribonucleoprotein enzyme that maintains the telomere length in a variety of organisms by the addition of TTAGGG repeats to the 3' ends of chromosomes (Morin, 1989). It consists of both RNA and protein catalytic subunits (Nakamura, 1997; Feng, 1995). The RNA component (TERC) is ubiquitious and has been found to be expressed in a wide variety of normal human tissues that do not exhibit telomerase activity (Feng, 1995; Avilion, 1996). The catalytic protein subunit that contains reverse transcriptase activity (hTERT), on the other hand, has a more limited expression profile that coincides with the presence of telomerase activity *in vivo* (Nakayama, 1998). Telomerase activity is normally found in human germ cells and in certain stem cells in rapidly

proliferating tissue (Kolquist, 1998). Telomerase activity is also found in a wide variety of human neoplasms and in some premalignant conditions (Kim, 1994; Kolquist, 1998).

Telomerase activation has been suggested to occur early in cancer progression. Clonal evolution, as proposed by Nowell, occurs by a process in which clones are generated and selected as a consequence of the ability to proliferate within the conditions present in an evolving neoplasm (Nowell, 1976). Only rare clones containing the appropriate genetic characteristics are able to adapt to the changing local environment, expand, and become an invasive cancer. Because the accumulation of the appropriate combination of genetic changes that confer malignancy occurs over a period of time, it has been proposed that cellular senescence may be an anti-cancer mechanism which works by limiting the number of divisions a cell can undergo. This reduces the likelihood that a cell will be able to undergo a genetic change that confers a malignant phenotype. In addition, continued cell division results in shortened telomeres that contribute to genetic instability due to the formation of sticky ends that promote end to end associations between chromosomes (Wan, 1999; Saltman, 1993). Cultured human fibroblasts at high passage levels (close to senescence) frequently contain chromosome aberrations, particularly dicentric and ring chromosomes, and dicentric chromosomes increase significantly in humans with age *in vivo* (Harley, 1991). Chromosomes with shortened telomeres are more subject to loss or rearrangement (Sandell, 1993). Replicative senescence prevents the propagation of cells with chromosomes that are likely to become unstable because of shortened telomeres. Interestingly, the human chromosome 17p arm (on which p53 is located) contains the shortest telomere in human cells and suggests that the inactivation of p53 in many human tumors may be partially related to telomere shortening in which end to end fusions involving 17p may give rise to breakage and loss of the 17p arm (Martens, 1998). The lengthening of telomeres by telomerase could conceivably reverse chromosome instability caused by shortened telomeres. This is important in cancer progression since too much instability leads to cell death as seen in cell cultures undergoing crisis. Immortalized SV40 or Ad5 transformed cells that survive crisis and exhibit telomerase activity show a stabilization in the frequency of dicentric chromosomes as compared to near-crisis cells

(Counter, 1992). Cytogenetic studies have suggested that acquisition of telomeric sequences can stabilize sites of chromosome breakage (Meltzer, 1993).

The effect of telomerase activation in normal human cell types has been examined by a number of investigators. Activation of telomerase by the forced expression of the reverse transcriptase catalytic subunit (hTERT) allows the extension of *in vitro* lifespan of normal human diploid fibroblasts (NHDF) and retinal pigment epithelial (RPE) cells (Bodnar, 1998). Expression of hTERT also confers lifespan extension in human keratinocyte or mammary epithelial cells, however, this requires the concomitant inactivation of the Rb/$p16^{INK4a}$ pathway (Kiyono, 1998). The activation of telomerase in both NHDF and RPE cells does not appear to change the growth factor requirements, cell cycle checkpoint responses, or DNA strand break rejoining activity originally found in the cells prior to hTERT expression (Jiang, 1999; Morales, 1999; Vaziri, 1999). Furthermore, activation of telomerase did not appear to change the chromosome stability or confer tumorigenic potential to hTERT expressing NHDF or RPE cells (Bodnar, 1998; Morales, 1999; Jiang, 1999), although there were some transient chromosome aberrations in hTERT expressing NHDF that appeared to be resolved with continued passaging (Vaziri, 1999). The maintenance of genetic stability in normal human cells by telomerase is not surprising, since normal cells possess the repair mechanisms and cell cycle checkpoints required to preserve genomic integrity. Furthermore, unless cells are at high passage levels, the telomere length should be sufficiently long that end to end associations and chromosome breakage are unlikely to occur.

Barrett's esophagus is a human premalignant condition in which the normal squamous cells lining the esophagus are replaced by metaplastic columnar epithelium which is predisposed to the development of esophageal cancer. The cell cycle and genetic abnormalities that occur in progression to cancer in Barrett's esophagus have been delineated through the examination of endoscopic biopsies. Elevation of 4N DNA content fractions and inactivation of p53 and p16 are early changes that contribute to cancer progression in this condition. There have been only two studies that have investigated telomerase activity in Barrett's esophagus. Telomerase activity has been reported in tissue obtained

from Barrett's esophagus patients—four out of eight patients examined showed telomerase activity as assayed by the telomerase repeat amplification protocol (TRAP) (Ozawa, 1997). The histologic grade of the tissues examined was not reported in this study. A different study using in situ hybridization techniques on tissue sections showed that 100% of the esophageal adenocarcinomas and high grade dysplasia samples contained high levels of telomerase RNA and 90% of low grade dysplasia samples contained moderate levels of telomerase RNA (Morales, 1998). In the same study, the basal crypt cells of metaplastic Barrett's esophagus contained weak to moderate levels of telomerase RNA in 70% of cases. Telomerase activity was not assessed in this study. The authors suggest that the increase in the presence of telomerase RNA in the transition to esophageal cancer may be indicative of the selection of an immortal population of metaplastic cells that are more prone to undergoing genetic changes due to continued proliferation (Morales, 1998).

Thus far, there have been no studies examining the effect of forced hTERT expression in premalignant, mortal but chromosomally altered human cells *in vitro*. Previous work examined the activation of telomerase in cells expressing either simian virus 40 large T antigen (SVLT), E6/E7 and/or H-ras (Halvorsen, 1999; Morales, 1999; Hahn, 1999) but not cells derived from premalignant tissue. Retroviral transfection of hTERT into late passage mortal SVLT-Ras^{val12}-transformed human pancreatic cell lines and SVLT-transformed human embryonic kidney cells prevented the onset of crisis (Counter, 1998; Halvorsen, 1999). Altered growth and morphology was observed in both of the hTERT expressing cultures, but the effect on the genetic stability in the cells was not examined. Co-expression of E6/E7 or H-ras with hTERT in NHDF did not confer tumorigenic potential as assessed by growth in soft agar (Morales, 1999). However, co-expression of SVLT and H-ras with hTERT in human embryonic kidney cells or human fibroblasts did cause an increase in tumorigenic potential as assessed by anchorage independent growth in soft agar and tumor formation in nude mice (Hahn, 1999).

We have previously established premalignant Barrett's cell strains that contain elevated 4N DNA content fractions as well as p53 and p16 inactivation (Palanca-Wessels, 1998). These cells do not

immortalize spontaneously and previous attempts to immortalize the cells with either repeated 4-nitroquinolone oxide treatment or expression of adenovirus E6 protein (in $p53^{+/+}$ Barrett's cell strain KR-42421) were not sufficient to extend lifespan of the cell strains (data not shown). We investigated the effect of telomerase activation in these mortal Barrett's cell strains by transducing the cells with a retroviral vector containing hTERT. We wanted to determine whether activation of telomerase would immortalize the Barrett's cell strains and whether this would lead to changes in the growth factor and anchorage dependency. We also tested the hypothesis that telomerase activity would reduce the chromosome instability present in premalignant Barrett's esophagus cells that contain early changes associated with neoplastic progression *in vivo*. We found that while telomerase activation did not, in general, cause overt changes in growth factor dependency (suggesting that the normal metabolic and signalling pathways in these cells are intact), the expression of hTERT did allow the acquisition of modestly enhanced anchorage independent growth. Expression of hTERT did not cause gross ploidy changes, with one exception, and slightly reduced the 4N fraction present in the cultures. Furthermore, hTERT expression did not change the level of genetic instability that was already present in the Barrett's cell strains.

Results

Extension of lifespan in Barrett's cell strains by expression of hTERT

In collaboration with James McDougall and Aloysius Klingelhutz, Barrett's esophagus cell strains (KR-42421; CP-52731; CP-94251; CP-18821) were infected with a retroviral construct containing the gene for human telomerase reverse transcriptase (hTERT) or the empty vector (LXSN) as described previously (Kiyono, 1998). Cell strains were each infected on two separate occasions for the selection of transduced clones or mass cultures. After infection, cells which had taken up the vector were selected for resistance to G418. The cultures were then either plated for clone selection or maintained as mass cultures. As a control, we also attempted to transduce normal esophageal squamous cells but were unable to select any transformants that lived longer than the parental cells (data not shown). This was consistent with the observation that immortalization of certain epithelial cells requires the inactivation of the p16/Rb pathway (Kiyono, 1998). We were, however, able to generate hTERT

transduced normal human diploid fibroblasts (82-6 hTERT) for use as a control for the genetic instablity studies described below. We found, as had been previously reported (Bodnar, 1998) that hTERT transduced fibroblasts acquired an extended lifespan, attaining a population doubling level (PDL) greater than 100, compared to parental fibroblasts (average maximum PDL 70). We also attempted the transduction of a primary explant culture from a Barrett's esophagus endoscopic biopsy, but this was not successful in establishing an immortal Barrett's epithelial culture.

In collaboration with Aloysius Klingelhutz, telomerase activity was measured by the telomere repeat amplification protocol (TRAP) assay in the Barrett's esophagus non-transduced and hTERT transduced (hTERT+) mass cultures, as well as selected hTERT transduced clones. All hTERT+ cultures showed measurable telomerase activity whereas the parental Barrett's cell strains before transduction showed no activity. In the representative example shown in Figure 4.1, individual hTERT+ clones derived from KR-42421 showed different levels of telomerase activity ranging from low to high, whereas the culture transduced with empty vector (LXSN) showed no telomerase activity.

Barrett's cell strains have a finite proliferative lifespan, eventually senescing after extended culture and rarely proliferating beyond 30 PDLs (Palanca-Wessels, 1998). The hTERT+ clones and mass cultures were maintained and PDLs recorded at each passage in order to determine lifespan. In all cases, the hTERT+ cultures attained population doubling levels greatly in excess of the corresponding parental and/or LXSN transduced strain (Table 4.1 and 4.2). Cell strains were transduced with either hTERT or LXSN at the following PDLs for the selection of clones: KR-42421 (PDL 22); CP-52731 (PDL 14); CP-94251 (PDL 17); CP-18821 (PDL 13). Two hTERT+ clones from each of the four Barrett's cell strains were passaged extensively. All hTERT+ clones have attained greater than twice the normal total population doublings of the corresponding parental strain. No clones from LXSN transduced (LXSN+) cultures were able to proliferate beyond a total of 35 PDLs (range 22-35) whereas all individual hTERT transduced clones have attained at least 69 PDLs (range 69-99) (Table 4.1). In one case (CP-18821), no clones were obtained from the LXSN+

culture, presumably because of the late passage level of the parental strain at the time of infection (infected at PDL 14; average PDL for this strain at senescence=17). In another cell strain, KR-42421, infected at at late passage (PDL 22; average PDL for this strain at senescence=22), three clones were picked but none of these grew after selection. Cell strains also were transduced with either hTERT or LXSN at the following PDLs and maintained as mass cultures: KR-42421 (PDL 24); CP-52731 (PDL 18); CP-94251 (PDL 17); CP-18821 (PDL 13). Under mass culture conditions, LXSN+ cultures were unable to be passaged beyond a total of 30 PDLs (range 13-30) whereas hTERT+ cultures have been maintained for at least 66 PDLs (range 66-92) (Table 4.2). Thus far, no hTERT+ cultures have shown any indication of approaching senescence.

The morphology of hTERT+ cells was reminiscent of cells at early passage, in direct contrast to the LXSN+ cells at the same time following transduction (Figure 4.2). The hTERT+ cells were smaller in size with a large nucleus to cytoplasm ratio compared to the LXSN+ cells and showed a higher amount of proliferation in the cultures. The LXSN+ cells were enlarged and irregularly shaped, containing large vacuoles and dark granules, and a low nucleus to cytoplasm ratio. Even after undergoing population doublings far in excess of the normal maximum PDL of the parental strains, the hTERT+ cultures retained a cell morphology more similar to Barrett's cells at an earlier passage level. Moreover, the growth rate of hTERT+ cells has remained constant and has not shown any indication of reduction despite extended passaging (data not shown).

Growth factor requirements of hTERT expressing Barrett's cells

Although the parental Barrett's cell strains are not immortal, the cells have been shown to contain genetic changes, including p16 and p53 abnormalities, that occur early in neoplastic transformation *in vivo* (Palanca-Wessels, 1998). These cells do not have many other features commonly associated with transformed cell lines, as they retain stringent growth factor requirements and grow poorly, if at all, in agar. We wanted to determine whether activation of telomerase induced phenotypic characteristics of malignant transformation in the Barrett's cell strains.

Barrett's esophagus cell culture media contains a complex mixture of growth factor supplements including fetal bovine serum (FBS), bovine pituitary extract (BPE), epidermal growth factor (EGF), and insulin-transferrin-selenium (ITS). We compared the fastidiousness of Barrett's esophagus hTERT+ mass cultures with non-transduced parental mass cultures by growing the cells in media containing decreasing concentrations of FBS, BPE, EGF and ITS. At the time of the experiment, the PDL of the parental and hTERT mass cultures, respectively, were as follows: KR-42421 (PDL 22 and 74.7); CP-52731 (PDL 8.2 and 86.4); CP-94251 (PDL 17 and 66.3); CP-18821 (PDL 13 and 64). The cells were plated in the various media formulations, fed and grown until one of the wells was near-confluent, then harvested and counted. Comparison of growth between hTERT+ cells and parental cells showed similar dependency on media growth factors with a few exceptions (see Figure 4.3 and 4.4) In a few cases hTERT+ cells showed slightly reduced dependency on BPE (CP-52731 and CP-94251) or EGF (CP-18821). On the other hand, hTERT+ cells showed increased dependency on FBS (CP-18821), EGF (CP-94251 and KR-42421), or ITS (CP-94251). Reduction of FBS concentration greatly inhibited cell proliferation in all cultures, both parental and transduced. Activation of telomerase did not lead to an overall reduction of growth factor dependency.

We compared the ability of the parental and hTERT+ Barrett's cells to form colonies in semi-solid medium. Cells were plated in agar, grown for one month and then scored for colony formation by visual examination under phase microscopy. The presence of colonies was confirmed by staining the nuclei with a DNA binding dye (PicoGreen) and measurement of average colony size was made. The minimum size scored as a colony was approximately 20 μm in diameter, encompassing a minimum volume of approximately 4200 μm^3 (roughly the volume of 8 cells; 1 cell is about 10 μm in diameter and 525 μm^3 in volume). This is equivalent to three cell divisions. The results of the colony counts are shown in Figure 4.5. Parental non-transduced Barrett's cells formed the fewest colonies, as compared to the control ras-transformed DLD-1 colon carcinoma line or the fibrosarcoma line HT-1080. Among the non-transduced Barrett's cell strains, there was a gradation in the number colonies observed, with KR-42421 showing almost no growth in agar and CP-52731 forming the most

colonies. The hTERT+ cultures all formed 3- to 6-fold more colonies than their corresponding parental strains which was a statistically significant difference (two-tailed p=0.001; Wilcoxon signed rank test). The average size of colonies is shown in Table 4.3. The average size of the colonies in CP-18821 and CP-52731 hTERT+ wells was roughly 2-fold larger than in the respective parental non-transduced wells. The average colony size in CP-94251 hTERT+ wells was about the same as in the parental wells. The KR-42421 parental wells contained only small clusters of cells with, occasionally, a rare colony that could be scored, whereas the KR-42421 hTERT+ colonies possessed an average size of more than two-fold larger. In all cases, however, whether parental or hTERT+, the colonies formed by the Barrett's cell strains were not as numerous or as large as the DLD-1 colonies. DLD-1 formed, on average, four-fold more colonies than hTERT+ cultures except for CP-52731 hTERT+ culture in which the difference was much less (Figure 4.5). The average colony size in CP-18821 hTERT+ wells was approximately half the average size of the DLD-1 colonies. The average colony size in CP-52731 hTERT+ wells was about one third as large as the colonies in the DLD-1 wells.

Effect of hTERT expression on the genetic stability of Barrett's esophagus cell strains

In Barrett's esophagus *in vivo*, aneuploid populations can be detected late in cancer progression. We never observed flow cytometrically detectable aneuploidy arise spontaneously in the Barrett's esophagus cultures. Chromosomal instability (CIN), however, may precede the detection of aneuploidy, as has been shown in ulcerative colitis and cancers of the head and neck, lung, bladder, cervix and breast (Hittelman, 1996; Hittelman, 1993; Rabinovitch, 1999; Willenbucher, 1999). We were therefore interested in determining whether telomerase expression would induce ploidy changes or affect CIN in the hTERT+ cultures.

We analyzed the DNA content of parental and hTERT+ cells by flow cytometric methods using chicken erythrocyte nuclei (CEN) as a standard to determine ploidy (Table 4.4). In general, we found similar ploidy between hTERT+ and corresponding parental cultures by this method. However, in CP-18821 hTERT+ cells grown in mass culture, we found that in addition to a diploid population,

there was both a near-tetraploid aneuploid subpopulation and a significant tetraploid population (Figure 4.6). We re-analyzed the cells for ploidy on the flow cytometer at a later passage and found that the aneuploid peak was still present and therefore appeared to represent a stable population. This is the only example of *in vitro* generation of DNA aneuploidy in Barrett's esophagus cells that we have encountered.

We also analyzed the cell cycle distribution of the parental and hTERT+ cells (Table 4.5). In particular, we were interested in the proportion of 4N (G2/tetraploid) cells in the cultures since elevated 4N DNA content fractions *in vivo* are associated with centrosome abnormalities (Chapter 3), progression to aneuploidy (Galipeau, 1996) and cancer (Reid et al, submitted). If shortened telomeres contributed to the accumulation of cells containing a 4N DNA content, activation of telomerase might affect the proportion of cells in this ploidy fraction. All four parental Barrett's cell strains showed a high 4N fraction, ranging from 22.6% to 33.2% of the total cell population, consistent with our previous measurements of primary Barrett's esophagus cultures (see Chapter 2; Palanca-Wessels, 1998). The 4N fraction appeared to be reduced in all of the hTERT+ clones when compared to the corresponding parental culture. The 4N fraction was appreciably decreased in one of the hTERT+ mass cultures (CP-52731) but was elevated in two hTERT+ mass cultures (CP-94251 and CP-18821). The CP-18821 hTERT+ mass culture contained tetraploid and near-tetraploid populations as described above. The data suggested that while the effect of telomerase activation on the 4N fraction was variable, the percentage of cells with 4N DNA content generally remained relatively high.

Flow cytometric analysis cannot distinguish between diploid G2 and tetraploid G1 cells, both of which have a 4N DNA content. In order to determine whether telomerase activation affected the percentage of tetraploid cells in the parental and hTERT+ cultures, we used two-color fluorescence in situ hybridization (FISH). We hybridized pairs of FISH probes, one probe labelled with fluorescein isothiocyanate (green fluorescence) or Texas Red (red fluorescence), to 4N sorted cells of the parental and hTERT+ cultures. 4N sorted cells that exhibited 4 widely separated foci for both green and red probes (thus containing 4 copies of each chromosome) were scored as tetraploid. The FISH results

are shown in Table 4.6. In general, a reduction in the 4N fraction was associated with a reduction in the percentage of tetraploids in the 4N fraction and a decrease in the percentage of tetraploids in the culture overall.

To study the effect of telomerase activation on chromosomal stability in greater detail, we performed karyotypic analyses on the parental and hTERT+ cultures in collaboration with Drs. Tom Norwood and Kent Opheim. Karyotypic analyses allowed us to detect the full spectrum of structural and numerical chromosome alterations that might be present in the cultures. Table 4.7 summarizes the karyotypic data obtained from both Giemsa staining and spectral karyotyping (SKY) techniques. An example of a spectral karyotype of a cell from a hTERT+ CP-52731 clone is shown in Figure 4.7. A previous study using SKY did not show karyotypic instability in telomerase-expressing normal diploid human fibroblasts (Vaziri, 1999). We confirmed this finding using normal human fibroblast strain (82-6) and hTERT+ fibroblasts derived from this strain by retroviral transduction using the same protocol as was applied to the Barrett's cell cultures. The hTERT+ human diploid fibroblasts have achieved greater than 100 population doublings, as described above. Neither parental nor hTERT+ human diploid fibroblasts showed any karyotypic abnormalities by SKY or Giemsa band staining.

For the Barrett's cell strains, analysis was confined to only diploid cells although tetraploid metaphases were also occasionally observed. The non-transduced Barrett's cell strains exhibited chromosome instability as evidenced by non-clonal aberrations. The degree of instability, however, varied between the different cell strains. For example, KR-42421 contained minimal clonal karyotypic aberrations, whereas CP-18821 contained a number of clonal chromosome aberrations in addition to multiple non-clonal structural and numerical abnormalities. Interestingly, KR-42421 was the only cell strain derived from tissue obtained from Barrett's metaplasia rather than high grade dysplasia. This cell strain also did not contain 17p loss of heterozygosity or p53 mutation, although the p53-dependent radiation induced G1 arrest appeared to be compromised (Palanca-Wessels, data not shown). The presence of numerous aberrations in the CP-18821 parental line might be due to its

late passage level since cells close to senescence typically show increased chromosome abnormalities (Sakesela, 1963; Benn, 1976). In general, the hTERT+ clones contained the same clonal abnormalities as the corresponding non-transduced parental cell strain. The hTERT+ cells also possessed additional clonal and non-clonal abnormalities not present in the parental cultures. Because we examined hTERT+ clones, the non-clonal chromosome aberrations within these clonally derived cultures were an indication of ongoing genetic instability. The hypothesis that activation of telomerase may reduce genetic instability by eliminating the end to end associations that occur between chromosomes with shortened telomeres (and that can lead to chromosome bridge-breakage-fusion cycles) did not appear to be supported by these karyotypic observations in Barrett's esophagus cells.

Karyotypic analysis allowed us to detect chromosome aberrations in detail but was limited in the number of cells that could be examined. Fluorescence in situ hybridization (FISH) of interphase cells is more sensitive in detecting chromosomal instability due to the greater number of cells that can be examined and because some cells with aberrant chromosomes may not be able to enter mitosis; thus, karyotypic analysis may underestimate the frequency of CIN (Dohner, 1993). Interphase FISH has been used to assess the CIN phenotype in colon cancer cell lines (Lengauer, 1997) and ulcerative colitis (Rabinovitch, 1999). Therefore, to obtain a broader view of the effect of telomerase expression on chromosomal stability throughout the cell population, we used FISH to scan for chromosome copy number distribution in parental and hTERT+ cells (Table 4.8). We used probes specific to the centromeric region of chromosomes 3, 7, 8, and 18. The chromosomes that we examined were primarily chosen because of the availability of bright fluorescent probes, although we avoided using probes to chromosomes 9 and 17 since these chromosomes contain the two most common sites of LOH in Barrett's esophagus *in vivo* (Barrett, 1996a) and we did not want to bias the results by choosing chromosomes that are known to be lost preferentially. To avoid the complication of tetraploid cells which are present in the cultures and which would have made the analyses more difficult to interpret, we performed FISH on cells which had been flow sorted into a pure DNA

diploid population. We also performed FISH on parental and hTERT+ 82-6 normal human fibroblasts. Data are shown in Table 4.8.

Chromosome instability was present in the majority of the Barrett's esophagus cultures but not in the 82-6 parental and hTERT+ fibroblast mass cultures. The average variation in chromosome copy number (percent of cells with non-modal FISH counts) in the the parental and hTERT fibroblast cultures was, respectively, 1.1% and 1.5% and probably represents the background due to variability in probe hybridization and spot identification. In contrast, the average variation in chromosome copy number in the Barrett's parental mass cultures ranged from 7.8% to 19%. The average variation in the Barrett's hTERT+ mass cultures was similar to the Barrett's parental culture in two of cases (KR-42421 and CP-52731) and was reduced by approximately half in the other two Barrett's cultures (CP-94251 and CP-18821). In the Barrett's hTERT+ clones, variation was reduced in both clones of CP-18821 and CP-52731, and in one of the clones of KR-42421. There was no reduction in the variation observed in either of the Barrett's hTERT+ clones of CP-94251 and one of the KR-42421 hTERT+ clones. Results obtained from clones should not be compared to results obtained from mass cultures, since the latter consist of many, potentially heterogeneous clonal subpopulations. In the analysis of *clones*, there should be little, if any, variation in the chromosome copy number amongst the cells without the effect of chrosomal instability during clonal growth. The only Barrett's hTERT+ clone in which there appeared to be little chromosomal instability was CP-18821 clone #12 in which the average variation was reduced to 2.8% among the chromosomes examined. We were unable to examine parental clones because the parental strains were not clonogenic under any attempted culture condition. We were, however, able to grow short-lived LXSN+ clones from two of the cell strains (KR-42421 and CP-94251). The average chromosomal instability of these LXSN clones (Table 4.8) closely approximated the instability found in their respective parental mass cultures. The one exception was KR-42421 LXSN+ clone #1 which exhibited only half of the variation present in the parental culture. Therefore, the variation observed in the parental culture was unlikely to be solely due to polyclonal mass culture derivation, but appeared to be a manifestation of the genetic instability inherent in the cells.

With the help of Dr. Ziding Feng, we analyzed the FISH data to determine the effect of hTERT transduction on chromosome instability. We determined that the hTERT+ cultures had, on average, a significantly lower number of cells with non-modal FISH counts than non-hTERT+ cultures (mean percentages of non-modal cells are 7.7% for hTERT+ versus 12.5% in non-hTERT+, p-value =0.014), implying that telomerase activation in the Barrett's esophagus cultures reduced the amount of chromosome instability as assayed by FISH. Furthermore, the effect of hTERT on chromosome instability appeared to be chromosome non-specific (p-value=0.0005). We did find, however, that chromosomes 3 and 18 had a higher percent of non-modal cells compared to chromosomes 7 and 8 (p-value =0.007). Not surprisingly, we found that the mass cultures have a higher number of non-modal cells than clonally derived cultures regardless of hTERT status (11.9% versus 7.4%, p-value=0.028).

Discussion

Previous studies have examined the phenotype of hTERT immortalized normal human diploid fibroblasts, but there has been up till now no information on the effect of telomerase activation on genetically altered, but mortal, cells derived from premalignant tissue. We have described the effect of retroviral transduction of hTERT vector into premalignant Barrett's cell strains that do not contain telomerase activity and possess a finite lifespan. We found that hTERT expression greatly extended the lifespan of hTERT+ Barrett's cells as compared to the corresponding parental or empty vector (LXSN) transduced strain. Growth factor dependency of the hTERT+ cultures remained largely similar to the parental cultures, although there was a significant increase in the ability to grow in agar. We examined the effect of hTERT expression on the genetic stability of the cells by using flow cytometric and cytogenetic techniques. While hTERT expression did not (with one exception) alter the overall DNA content of the hTERT+ cultures compared to the corresponding parental cultures by flow cytometric analysis, there was a reduction in the proportion of cells in the 4N DNA content fraction in the majority of cultures. Although the underlying chromosomal instability (analyzed by both karyotypic and FISH analyses) present in the parental cultures was not eliminated by hTERT

transduction, the reduction in chromosome variability as assessed by FISH was statistically significant. This suggests that telomerase activation can enhance genomic stability.

Replicative senescence has been proposed to be a mechanism that protects against the evolution of cancer. By limiting the number of population doublings a cell is able to undergo, the probability that the correct combination of genetic changes will occur leading to tumor formation is greatly reduced. Our data supports this hypothesis. We find that, unlike in normal human diploid fibroblasts, activation of telomerase in Barrett's esophagus cells enhanced the colony forming efficiency of cells to grow in agar. The only other report where anchorage independent growth was observed following hTERT transduction was in human embryonic kidney cells and fibroblast co-expressing SVLT and H-ras (Hahn, 1999). Since our experiments were performed some time after introduction of the hTERT vector, we cannot say that activation of telomerase per se directly caused a change in the ability of the Barrett's epithelial cells to grow in agar. The continued chromosome instability after telomerase activation, as shown by SKY and FISH, may have contributed to the evolution of cells with reduced anchorage dependency for growth. The clones used in the anchorage independent growth assay had undergone at least 40 population doublings after transduction with hTERT. Testing the cells soon after telomerase activation, both for changes in growth factor dependency or gene expression changes would be one way of determining whether changes observed were a primary or a secondary effect of telomerase activation. Alternatively, the increase in the number of colonies on agar may be due to an enhancement in the growth rate of hTERT+ cells compared to their parental counterparts. During the assay period, the hTERT+ cells may have been able to undergo more population doublings than the parental cells and thus formed more colonies of scorable size. The high passage level of two of the parental cell strains (KR-42421 and CP-18821) and accompanying slow down of growth may have limited the ability of the cells to form colonies. The growth period of the assay, however, was one month and during this time most cells should have been able to undergo the three population doublings required for a colony to reach scorable size (see Results section).

Continued passaging of the hTERT+ cells may eventually lead to reduced growth factor dependency that we were not able to see at the time we performed these experiments. Continued genetic evolution of the hTERT+ cells with extended passaging appears to be occuring. Gross aneuploidy that arose in one hTERT+ mass culture (Figure 4.6) may have arisen as a consequence of continuing chromosome instability during the extended lifespan of the cells as opposed to a direct effect of hTERT itself. This hTERT+ culture represents the only observed aneuploid population that we have observed during culture of Barrett's esophagus epithelium. We cannot say whether the aneuploid population in the CP-18821 hTERT+ culture represents a pre-existing sub-population that was immortalized at the time of transduction with hTERT or emerged during evolution *in vitro*. It does appear, however, that this population could not have evolved to comprise a large percentage of the culture in the absence of the extended lifespan of these cells. How closely this may parallel neoplastic evolution *in vivo* (in which aneuploidy follows incrased 4N fractions by an average of 17 months (Galipeau, 1996) and usually precedes cancer (Rabinovitch, 1988). It is known, however, that adenocarcinoma arising in Barrett's esophagus uniformly expresses high levels of telomerase RNA (Morales, 1998).

Our work suggests that an important effect of activation of telomerase in neoplastic progression is to extend lifespan in cells that are already be genetically unstable and thus allow further cell division and evolution to a malignant phenotype. While stabilization of telomere length may be necessary, it is not sufficient for progression to cancer; without genetic instability, neoplastic evolution will not occur as evidenced by the absence of cancer-associated changes in normal "immortalized" human cells expressing telomerase (Bodnar, 1998; Morales, 1999; Vaziri, 1999). Too much genetic instability, however, is detrimental to the continued proliferation of cells and may lead to a high rate of cell death. A previous study had suggested that telomerase activation in immortalized SV40 or Ad5 transformed cells surviving crisis stabilized the frequency of dicentric chromosomes in the cultures compared to the pre-crisis state (Counter, 1992). Our data also suggest that activation of telomerase can reduce the amount of genetic instability present in a cell, although it does not eliminate it. Other

mechanisms, such as centrosome amplification (Chapter 3), could contribute to instability and would not be affected by telomerase activation.

Besides providing insight into the contribution telomerase makes in neoplastic progression, the immortalization of the Barrett's cell strains allows us to conduct experiments which were impossible to perform due to the finite lifespan and limited cell number of these cultures. These potentially include expression array experiments in which large cell numbers are required in order to obtain sufficient RNA to interrogate an expression chip. The observation that the overall growth factor requirements of the hTERT+ cells remains similar to the non-transduced cells suggests that the cells can be used for the study of metabolic and cell-signalling pathways in Barrett's esophagus cells. The hTERT+ cells could also be used as an *in vitro* model of progression. It would be interesting to monitor the changes that occur with continued passaging and whether these reiterate what is observed *in vivo* at both the gross ploidy and genetic levels. An *in vitro* organotypic system utilizing hTERT+ cells to mimic progression *in vivo* could also be used to test the possible contribution of carcinogens (e.g. bile acids or nitrosamines) to neoplastic progression *in vivo* or to test promising therapeutics aimed at treating or halting the development of cancer (see Chapter 5).

Methodology

Cell strains and cell culture conditions

CP-52731, CP-94251, CP-18821, and KR-42421 cell strains established from Barrett's esophagus biopsies and were maintained in modified MCDB 153 as described previously (Chapter 1; Palanca-Wessels 1998). 82-6 normal human fibroblasts were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS). DLD-1 colon carcinoma cell line and HT1080 fibrosarcoma line were maintained in RPMI containing 16% FBS and DMEM containing 10% FBS respectively. Cultures were kept in a humidified atmosphere containing 5% CO2 at 37°C and fed three times a week.

Viral construct and selection of transduced cells

Retroviral transduction was perfomed using a retroviral construct containing the gene for human telomerase reverse transcriptase (hTERT) or the empty vector (LXSN) as described previously (Kiyono, 1998). Transduced cells were selected for resistance to G418 and were either plated for clone selection or maintained as mass cultures.

Telomere repeat amplification protocol (TRAP) assay

The telomere repeat amplification protocol (TRAP) assay was performed with several modifications to the protocol described originally (Kim, 1994) to control for false positives and inhibitors of PCR amplification. Cells were counted and lysed in CHAPS lysis buffer at a concentration between 12,000 and 20,000 cells per µl buffer and one µl was used for the TRAP assay. This number of cells was well within the range of exponential amplification of products in the TRAP assay using positive control cells that had high telomerase activity. The TS primer was kinase labeled with g-ATP and used at 25 x 10^6 cpm/µg. The CX-ext primer is a modification of the original CX primer and was designed to prevent primer dimer interactions and false positives (Krupp, 1997). An internal ITAS fragment was included to control for the PCR reaction (Wright, 1995). PCR cycles was carried out as described (Kim, 1994) and one-eighth of the sample was run on a 7.2% denaturing polyacrylamide gel. The gel was dried and exposed to XAR-5 film overnight. Relative telomerase signal intensity was measured by phosphor imaging analysis (Johnston, 1990).

Growth factor requirement assay

3 x 10^4 cells were each seeded in individual wells of a 24-well tissue culture plate in media containing either 0%, 10%, 50% or 100% of the normal concentration of the growth factor to be tested (bovine pituitary extract, fetal bovine serum, insulin-transferrin-selenium, epidermal growth factor). Each condition was tested in triplicate wells. Cells were grown and harvested before 100% confluency, from seven to twelve days post-seeding. Cells were suspended in a small volume of media and counted visually using a hemocytometer.

Anchorage independent growth assay

Approximately 3.4 x 10^3 cells were suspended in 0.35% SeaKem GTG agarose (FMC Bioproducts, Rockland ME) in MCDB media and 5% fetal bovine serum over a 0.5% DNA grade agarose base layer in 6-well tissue culture plates. Cells were fed twice weekly with 2 mls of growth media. Colonies were scored visually under phase contrast microscopy by counting five random fields per well (four quadrants and the center) and then extrapolating the number of colonies present. Colonies needed to be at least 20 μm in diameter (volume 4200 μm^3) in order to be counted (approximately equivalent to a cluster of eight cells; estimated diameter of one cell is 10 μm for a volume of about 523 μm^3). This is equivalent to at least three cell divisions. Volume was estimated on the basis of positive nuclear PicoGreen staining (Molecular Probes, Eugene OR) of DNA using fluorescence microscopy. The diameter using light microscopy was 50-100% larger due to refractive effects of agar and addtional cytoplasmic volume.

Cytogenetics

Colcemid (Gibco, Grand Island, NY), 0.2 μg/ml, was added 24 to 37 hours prior to harvest for cytogenetic analysis. Cells were then incubated in hypotonic solution (potassium chloride and sodium citrate dihydrate) for 20 minutes at 37°C prior to being fixed in methanol: glacial acetic acid (3:1). Slides were prepared and G-banded according to standard cytogenetic methods. Structural chromosome abnormalities were considered clonal if the alterations were exhibited in at least two cells from a culture. Numerical chromosome abnormalities were considered clonal if observed in at least three cells from a culture. (*Get SKY method or ref.)

Flow cytometry

DNA content flow cytometric sorting was used to purify different ploidy populations from both *in vivo* and *in vitro* samples using a Coulter Elite cell sorter (Miami, FL). For viable sorting of cultured cells, cells were incubated in growth medium containing Hoescht 33342 (Calbiochem, La Jolla, CA) at 10 μM concentration for 30 minutes at 37°C. Cells were trypsinized after incubation, resuspended

in new growth media containing dye, and kept covered on ice until sorting. For DNA content analysis, cultured cells were trypsinized and resuspended in 10 μg/ml DAPI in NST buffer [146 mM NaCl, 10 mM Tris base, 0.1% NP40 (Sigma), 2 mM Ca, 20 mM Mg, and 0.05% BSA, pH 7.4] before analysis. BioSure Controls chicken erythrocyte nuclei (Riese Enterprises, Grass Valley, CA) was added as a standard to each sample prior to running on the flow cytometer. The Multiplus software program (Phoenix Flow Systems, San Diego, CA) developed by Dr. Peter S. Rabinovitch was used for cell cycle analysis.

Table 4.1 Summary of hTERT+ and LXSN+ Barrett's cell growth (clonogenic conditions)
For hTERT clone data, the highest PDL of any clone of a particular strain is noted. hTERT clones have not shown any cessation or reduction in growth. *denotes no further growth observed in the culture.

Parental non-transduced			*hTERT transduced clones*		*LXSN transduced clones*	
Cell strain	*Average max. PDL*	*PDL at infection*	*# clones picked*	*highest total PDL*	*# clones picked*	*highest total PDL*
KR-42421	24	22	16	95	3	22*
CP-52731	28	14	10	99	14	30*
CP-94251	29	17	17	69	20	35*
CP-18821	17	13	19	92	0	no clones

Table 4.2 Summary of hTERT+ and LXSN+ Barrett's cell growth (mass culture conditions)
For hTERT data, the highest PDL of the mass culture is noted. hTERT mass cultures have not shown any cessation or reduction in growth. *denotes no further growth observed in the culture.

Parental non-transduced			*hTERT transduced highest total PDL*	*LXSN transduced highest total PDL*
Cell strain	*Average max. PDL*	*PDL at infection*		
KR-42421	24	24	78	26*
CP-52731	28	18	92	no data
CP-94251	29	17	66	30*
CP-18821	17	13	70	13*

Table 4.3 Average volumes of colonies of parental non-transduced and hTERT+ Barrett's cells grown in agar. Volumes were calculated using measurements of colonies stained with PicoGreen. The volume of DLD-1 colon cancer line is also included

Cell Strain	*Average colony size (μm^3)*	
	Parental non-transduced	*hTERT transduced*
KR-42421	1685	2803
CP-52731	4251	8615
CP-94251	2685	2744
CP-18821	6645	13312
DLD-1	28094	not applicable

Table 4.4 DNA content analysis of parental (non-transduced) and hTERT+ Barrett's cells using CEN standard
*significant tetraploid and near-tetraploid aneuploid populations were observed in this culture.

Cell Strain	*Non-transduced mass culture*	*hTERT clone 1*	*hTERT clone 2*	*hTERT mass culture*
KR-42421	2.55N [PDL 22]	2.63N [PDL 70.2]	2.73N [PDL 82.7]	2.66N [PDL 74.6]
CP-52731	2.30N [PDL 14]	2.25N [PDL 13.1]	2.29N [PDL 74.2]	2.29N [PDL 76]
CP-94251	2.26N [PDL 17]	2.25N [PDL 61.3]	2.17N [PDL 68.8]	2.17N [PDL 58.6]
CP-18821	2.49N [PDL 13]	2.37N [PDL 63.7]	2.39N [PDL 56.3]	2.42N; 4.04N;4.56N* [PDL 53.8]

Table 4.5 4N fraction of parental (non-transduced) and hTERT+Barrett's cells

Cell strain	*Non-transduced mass culture*	*hTERT clone 1*	*hTERT clone 2*	*hTERT mass culture*
KR-42421	33.2% [PDL 22]	24.6% [PDL 70.2]	32.3% [PDL 82.7]	28.6% [PDL 74.6]
CP-52731	23% [PDL 14]	13.1% [PDL 13.1]	8.7% [PDL 74.2]	10.8% [PDL 76]
CP-94251	22.6% [PDL 17]	13.5% [PDL 61.3]	13.5% [PDL 68.8]	27.3% [PDL 58.6]
CP-18821	30.5% [PDL 13]	24.9% [PDL 63.7]	17.5% [PDL 56.3]	28.5% 4.04N; 44.6% 4.56N [PDL 53.8]

Table 4.6 Percentage of tetraploid cells in parental (non-transduced) and hTERT+ Barrett's cells
The percentage of tetraploid cells (as determined by FISH) in the sorted 4N population; the percentage of tetraploid cells in the total cell culture population is indicated in parentheses.

Cell strain	*Non-transduced mass culture*	*hTERT clone 1*	*hTERT clone 2*	*hTERT mass culture*
KR-42421	20.8% (total: 6.9%)	20.3% (total: 5.0%)	27.5% (total: 8.8%)	25.0% (total: 7.2%)
CP-52731	11.5% (total: 2.6%)	5.3% (total: 0.7%)	2.8% (total: 0.2%)	12% (total: 1.3%)
CP-94251	24.3% (total: 5.5%)	12.0% (total: 1.6%)	21.3% (total: 2.9%)	29.5% (total: 8.1%)
CP-18821	29.0% (total: 8.8%)	8.8% (total: 2.2%)	15.8% (total: 2.8%)	52.8% (total: 38.6%)

Table 4.7 Karyotypes of parental and hTERT+ cells
Numbers within the brackets represent the number of cells containing the listed karyotype. If the karyotype is the composite from a number cells, this is indicated by the number of cells is prefixed with cp. The clonal aberrations that are not present in the parental culture but are found in the corresponding hTERT+ culture are highlighted in bold type. Best estimates of chromosome breakpoints based on Giemsa banding are listed in the chapter notes at the end of chapter 4.

Cell culture	*Population doublings*	*Karyotype (ICSN diagnoses)*
82-6 mass culture	38	46, XY [5]
82-6 mass culture (hTERT+)	89	46, XY [5]
KR-42421 mass culture	20	47-53, XY, +4, +5, +9, +20, +0-3 markers [cp10]
KR-42421 clone #7 (hTERT+)	82	46-48, XY, **dup(8)**, +20 [cp5]
KR-42421 clone #19 (hTERT+)	70	47-48, XY, **+der(1)t(1;6)**, +20 [8]/ 94, XXY, **-Y, i(8)**, +9, **-12, -18**, +3 markers [1]
CP-52731 mass culture	15	43-45, XY, der(6)t(6;9;12), der(9)t(9;14), -12, der(14)t(6;14), der(17)t(1;17), del(21q) [cp5]
CP-52731 clone #1 (hTERT+)	99	44-46, XY, **-4**, der(6)t(6;9;12), der(9)t(9;14), -12, der(14)t(6;14), der(17)t(1;17), del(21q), **+ 1 marker** [cp15]
CP-52731 clone #8 (hTERT+)	91	44-46, XY, der(6)t(6;9;12), der(9)t(9;14), -12, der(14)t(6;14), der(17)t(1;17), **+18**, del(21q) [cp7]
CP-94251 mass culture	21	43-46, XY, der(9)t(9;17), der(10)t(10;17), -17, -21, +ring , +1-2 markers [cp10]
CP-94251 clone #2 (hTERT+)	64	42-45, XY, **-4, -9, -14**, der(9)t(9;17), -17, -21 [cp12]
CP-94251 clone #11 (hTERT+)	56	43-47, XY, der(9)t(9;17), -17, -21, +ring, +1-2 markers [cp8]
CP-18821 mass culture	14	39-40, X, -Y, der(1)t(1;22), der(17)t(17;20), -21, -22, multiple non-clonal structural (frequently complex) abnormalities, numerical abnormalities, and telomeric associations [cp14]
CP-18821 clone #10 (hTERT+)	86	43-45, XY, der(1)t(1;22), **del(2), der(10)t(10;15), -15**, der(17)t(17;20), **inv dup(22)** [cp11]
CP-18821 clone #12 (hTERT+)	52	44-46, XY, der(1)t(1;22), **der(3)t(3;10),dup(6), -10, der(14;15)**, der(17)t(17;20), **-19, der(21)t(3;21), inv dup(22)** [cp10]

Table 4.8 Chromosome instability in parental, hTERT+ and LXSN+ cells as assayed by FISH
FISH analysis was performed on 2N sorted cells using α-centromeric probes to chromosomes 3, 7, 8 and 18. At least 200 cells were counted for each probe, except for LXSN transduced short-lived clones where 100 cells were counted. A copy number was considered modal if at least 30% of cells possessed that number of chromosomes. The hTERT+ cultures contained, on average, a significantly lower number of non-model cells than non-hTERT+ cultures (p-value = 0.014). Abbreviations: Chr., chromosome; S.E.M., standard error of the mean; mass, mass culture; N.D., not done

	% cells varying from modal number(s) [modal copy number(s)]				*Average variation (non-modal cells) ± S.E.M.*
Cell culture	*Chr. 3*	*Chr. 7*	*Chr. 8*	*Chr. 18*	
82-6 mass (parental)	2% [2]	0.5% [2]	0% [2]	2% [2]	1.1% ± 0.5%
82-6 mass (hTERT+)	1.5% [2]	1% [2]	0.5% [2]	3% [2]	1.5% ± 0.5%
KR-42421 mass (parental)	9% [2]	7% [2,3]	6.5% [2]	8.5% [2]	7.8% ± 0.6%
KR-42421 mass (hTERT+)	4.5% [2]	8% [2]	2.5% [2]	26% [2]	10.3% ± 5.4%
KR-42421 clone #1 (LXSN)	N.D.	3% [2]	N.D.	4% [2]	3.5% ± 0.5%
KR-42421 clone #2 (LXSN)	N.D.	7% [2]	N.D.	17% [3]	12.0% ± 5.0%
KR-42421 clone #3 (LXSN)	N.D.	7% [2]	N.D.	7% [2]	7.0% ± 0.0%
KR-42421 clone #7 (hTERT+)	7% [2]	4% [2]	5.5% [2]	6.5% [2]	5.8% ± 0.7%
KR-42421 clone #19 (hTERT+)	10% [2]	4% [2]	6% [2]	5.5% [2]	6.4% ± 1.3%
CP-52731 mass (parental)	13% [2]	12% [2]	6% [2]	7.5% [2]	9.6% ± 1.7%
CP-52731 mass (hTERT+)	5.5% [2]	7.5% [2]	8.5% [2]	16.5% [2]	9.5% ± 2.4%
CP-52731 clone #1 (hTERT+)	4.5% [2]	4.5% [2]	4.5% [2]	10% [2]	5.9% ± 1.4%
CP-52731 clone #8 (hTERT+)	5.5% [2]	5.5% [2]	8.5% [2]	4% [2,3]	5.9% ± 0.9%
CP-94251 mass (parental)	19% [2]	11.5% [2]	4% [2]	10.5% [2]	11.3% ± 3.1%
CP-94251 mass (hTERT+)	4.5% [2]	3.5% [2]	6% [2]	8% [2]	5.5% ± 1.0%
CP-94251 clone #1 (LXSN)	N.D.	6% [2]	N.D.	20% [2]	13.0% ± 7.0%
CP-94251 clone #2 (LXSN)	N.D.	7% [2]	N.D.	13% [2]	10.0% ± 3.0%
CP-94251 clone #2 (hTERT+)	10% [2]	4% [2]	3% [2]	10% [2]	6.8% ± 1.9%
CP-94251 clone #11 (hTERT+)	15.5% [2]	2.5% [2]	5.5% [2]	13% [2]	9.1% ± 3.1%
CP-18821 mass (parental)	24.5% [2]	18% [2]	16% [2]	17.5% [2]	19.0% ± 1.9%
CP-18821 mass (hTERT+)	12.5% [2]	7% [2]	8.5% [2]	9% [2]	9.3% ± 1.2%
CP-18821 clone #10 (hTERT+)	3.5% [2]	2.3% [2]	2.8% [2]	2.5% [2]	2.8% ± 0.3%
CP-18821 clone #12 (hTERT+)	18.5% [2]	1.5% [2]	4% [2]	5% [2]	7.3% ± 3.8%

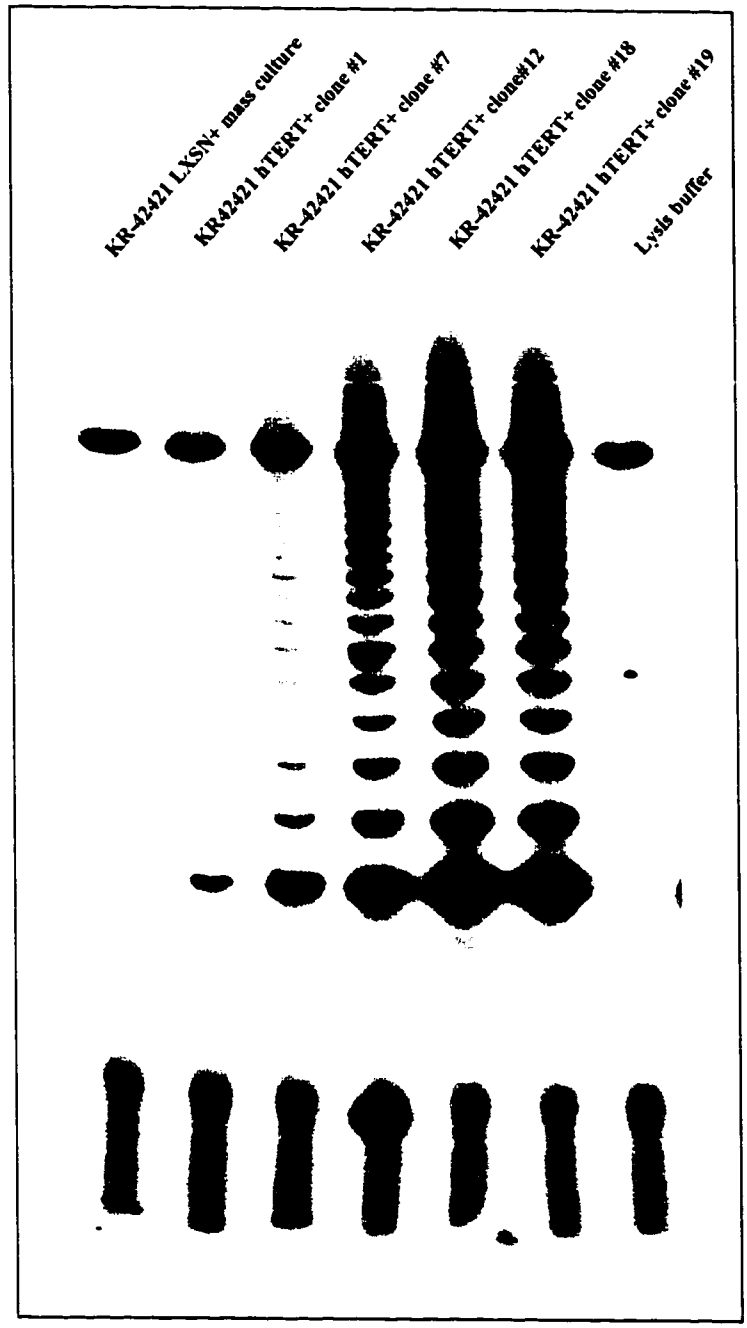


Figure 4.1 TRAP results of LXSN+ and hTERT+ Barrett's cells. Cell lysates from KR-42421 LXSN+ (Lane 1) and five separate hTERT+ clones (Lanes 2 through 6) were analyzed by the TRAP assay for telomerase activity. Lane 7 is lysis buffer (negative control). There is a difference in the level of telomerase activity present amongst the clones.

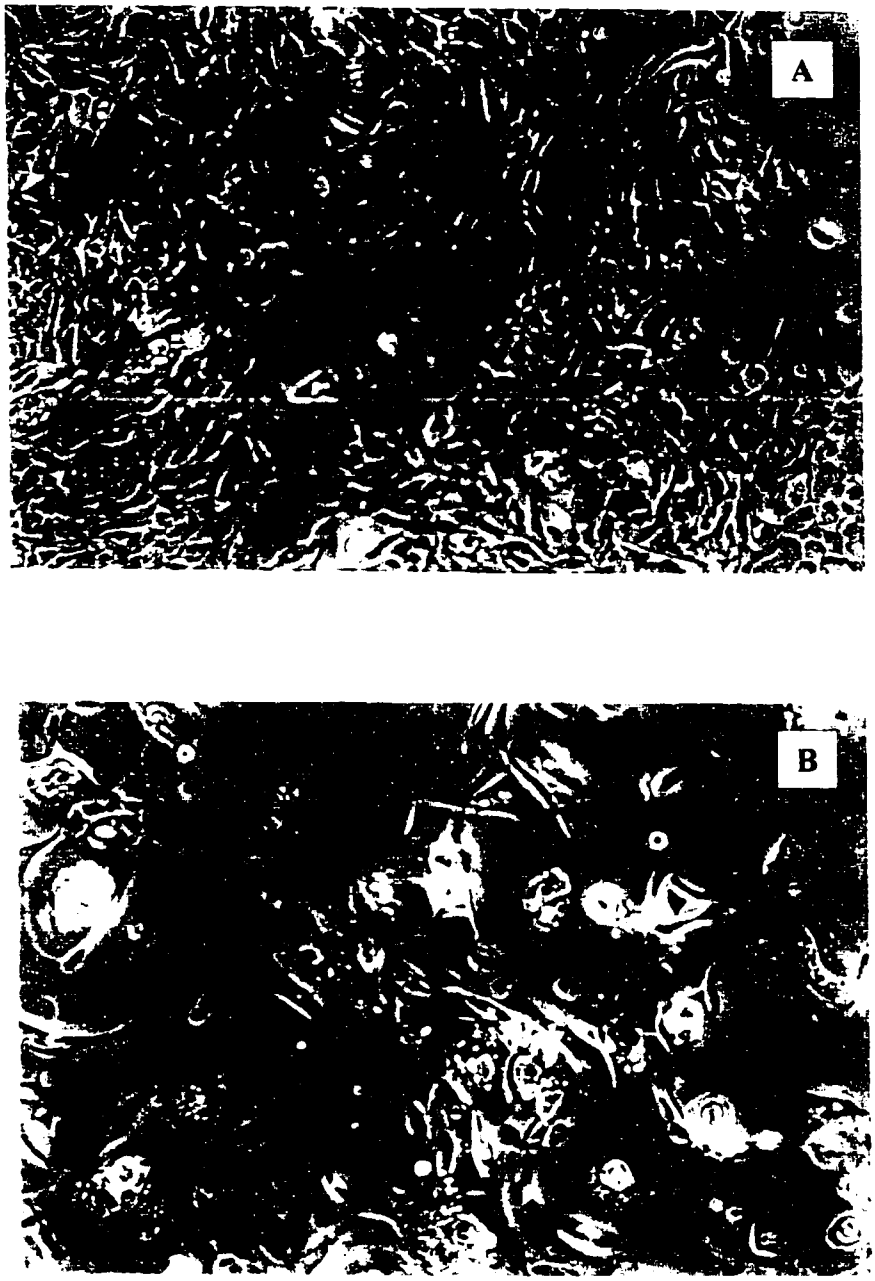


Figure 4.2 Phase-contrast microscopy of LXSN and hTERT transduced Barrett's esophagus cells. The KR-42421 hTERT+ mass culture (A) contains smaller cells and shows greater proliferation compared to the KR-42421 LXSN transduced mass culture (B) which contains large, irregularly shaped cells and little growth. These photographs were taken of both cultures at the same time following transduction.

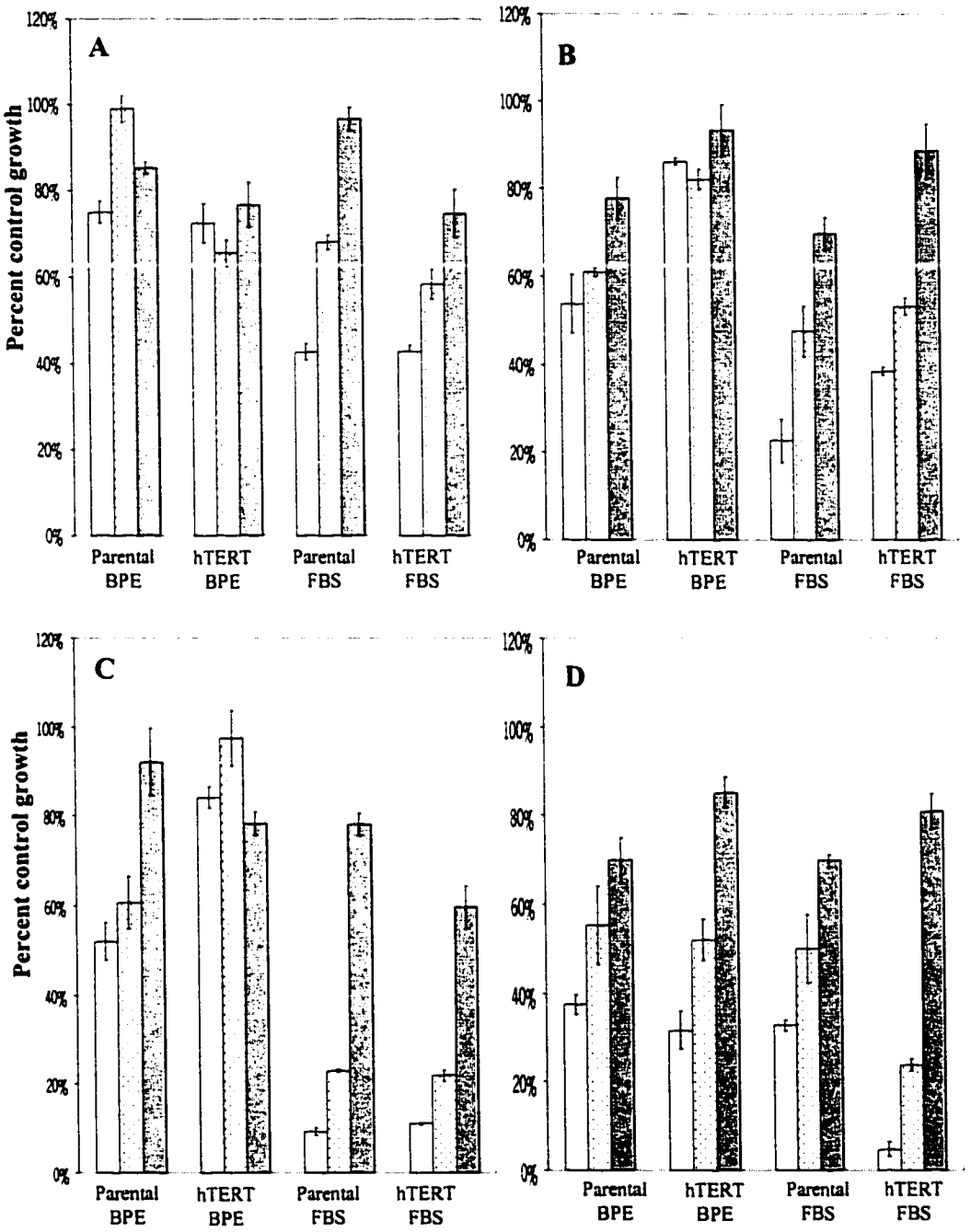


Figure 4.3 Growth factor dependency of parental and hTERT+ cells (BPE and FBS). Parental and hTERT+ Barrett's cell strains were grown in varying concentrations of BPE and FBS (*A*, KR-42421; *B*, CP-52731; *C*, CP-94251; *D*, CP-18821). Percent growth compared to control for cells grown in 0% (white bar), 10% (dotted bar), and 50% (gray bar) of normal growth factor concentration are shown. Y-error bars represent standard error of the mean for triplicate samples. See text for population doubling levels for the cells at the time of the experiment

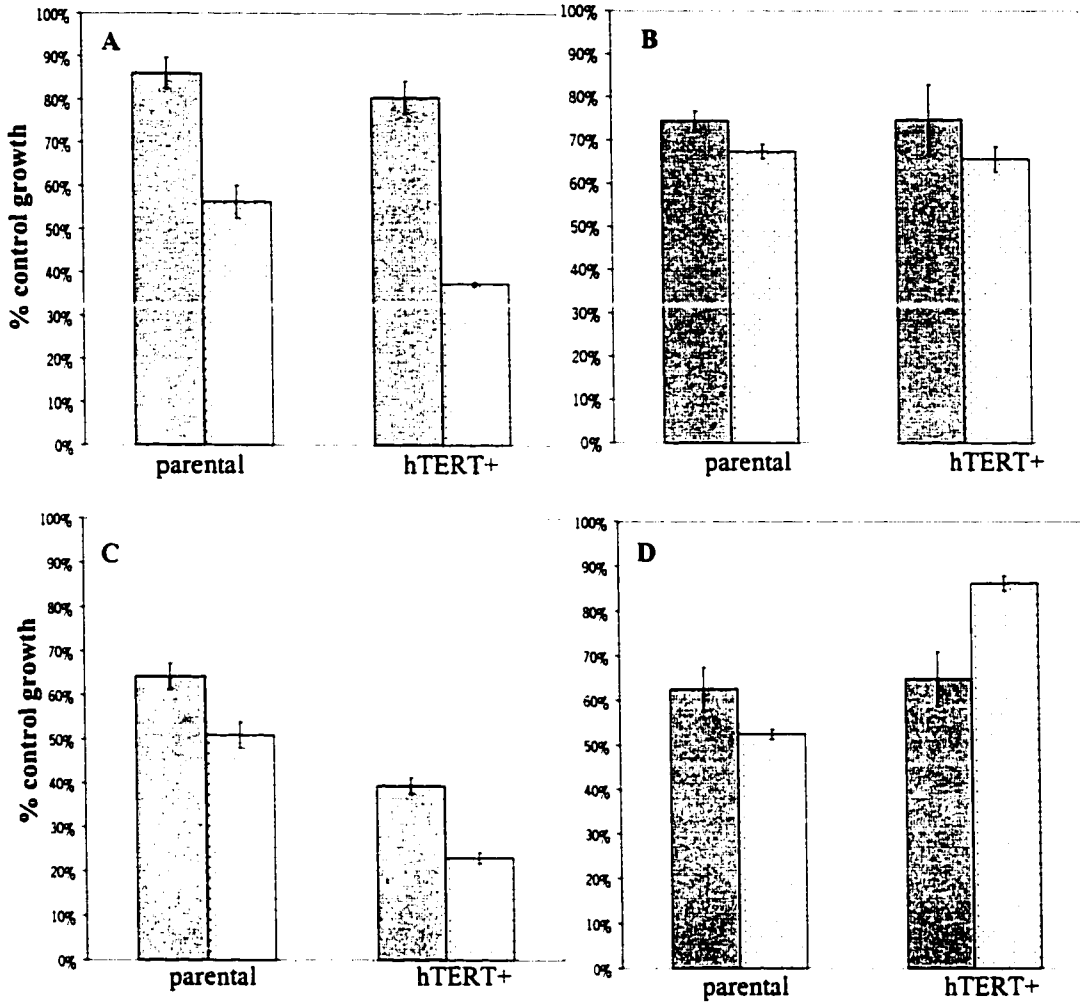


Figure 4.4 Growth factor dependency of parental and hTERT+ (ITS and EGF). Parental and hTERT+ mass cultures were grown in 50% of the normal concentration of ITS or EGF (*A*, KR-42421; *B*, CP-52731; *C*, CP-94251; *D*, CP-18821). Percent of growth compared to control is shown for ITS (dark gray bar) and EGF (light gray bar). Y-error bars represent standard error of the mean for triplicate samples. See text for the population doubling levels of the cells at the time of the experiment.

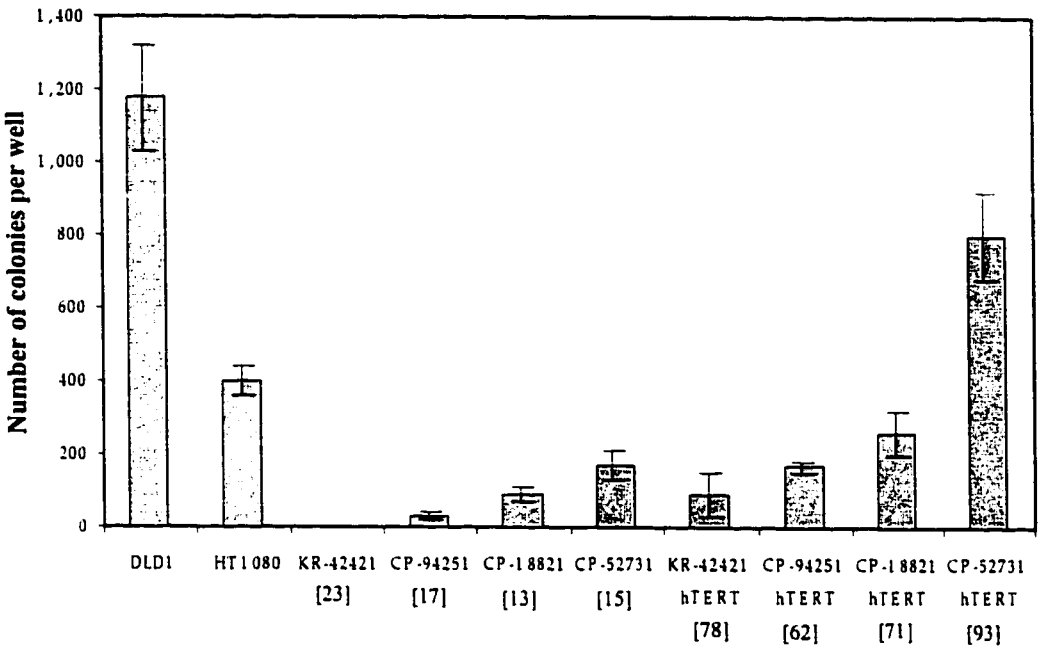


Figure 4.5. Anchorage independent growth of parental and hTERT transduced Barrett's esophagus cell strains. 3.4 x 10^6 cells from parental non-transduced Barrett's strains (KR-42421; CP-94251; CP-18821; CP-52371), hTERT transduced Barrett's strains (hTERT), one ras-transformed colon carcinoma line (DLD-1) and one fibrosarcoma line (HT1080) were plated in agarose coated plates. The population doubling levels of the parental and hTERT+ Barrett's cells at the time of plating are listed within the brackets. After one month, the number of colonies per well was scored. Error bars represent the standard deviation of triplicate plates. The difference in the number of colonies in the parental versus the hTERT wells was statistically significant (two-tailed p=0.001, Wilcoxon signed rank test).

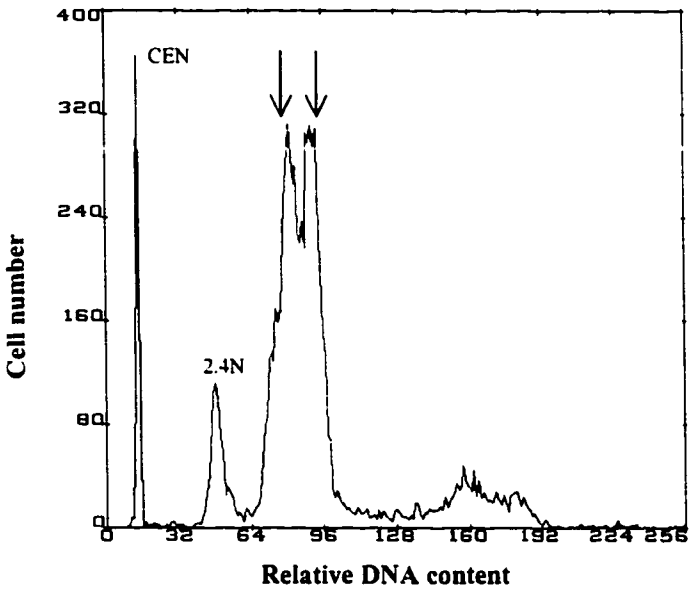
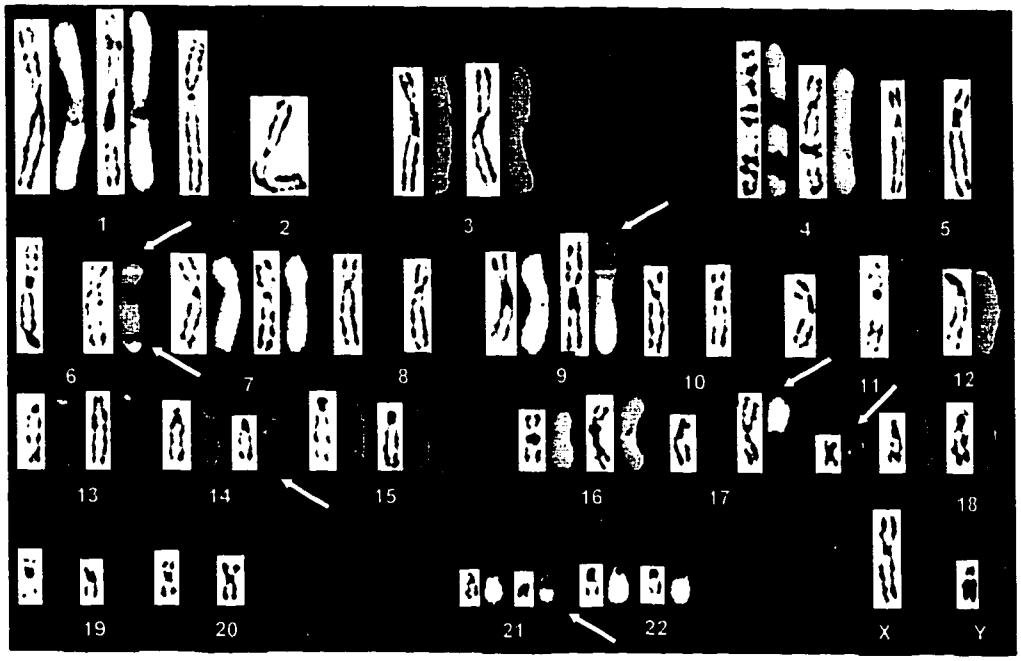


Figure 4.6 DAPI profile of CP-18821 hTERT+ mass culture. Chicken erythrocyte nuclei (CEN) were added to DAPI stained cells prior to being run on the flow cytometer. CEN possess approximately one-third of the DNA content of normal human diploid cells. There are two large peaks (indicated by the arrows) in the tetraploid range (approximately 4.0N and 4.6N).

CP52731-1 PDL 85



Diagnosis: **46,XY,der(6)t(6;9;12),der(9)t(9;14),**
-12,der(14)t(6;14),der(17)t(1;17),+?i(18p),del(21)

Figure 4.7 Spectral karyotype of a cell from a hTERT+ CP-52731clone. Spectral karyotyping enables the identification of complex chromosomal rearrangements not otherwise detectable by conventional Giemsa banding. Each chromosome is represented by a different color. The white arrows point to chromosome abnormalities listed in the diagnosis above. A Giemsa banded karyotype of both diploid and tetraploid non-transduced cells from the CP-52731 cell strain was shown in Figure 2.2. The unidentified marker chromosome can now be identified as a three-way chromosome rearrangement between chromosomes 6, 9 and 12.

Notes to Chapter 4

Estimates of the chromosome breakpoints of chromosome abnormalities found in the Barrett's parental and hTERT transduced cultures (Table 4.6) were made on the basis of Giemsa banding. Only cell strains for which selected abnormalities were analyzed are listed below. For complete karyotypes, refer to Table 4.6.

Cell strain	Estimated chromosome breakpoints
KR-42421 clone #19 (hTERT+)	der(1)t(1;6)(?p?12;?p?21), i(8)(q10)
CP-52731 mass culture	der(6)t(6;9;12)(12pter→12p?11.2::6p?11.2→6q?13::9?→9?), der(9)t(9;14)(p?12;q?13), der(14)t(6;14)(p12q?11.2), der(17)t(1;17)(?p31;q11.2), del(21)9q22
CP-52731 clone #1 (hTERT+)	der(6)t(6;9;12)(12pter→12p?11.2::6p?11.2→6q?13::9?→9?), der(9)t(9;14)(p?12;q?13), der(14)t(6;14)(p12q?11.2), der(17)t(1;17)(?p31;q11.2), del(21)9q22
CP-52731 clone #8 (hTERT+)	der(6)t(6;9;12)(12pter→12p?11.2::6p?11.2→6q?13::9?→9?), der(9)t(9;14)(p?12;q?13), der(14)t(6;14)(p12q?11.2), der(17)t(1;17)(?p31;q11.2), del(21)9q22
CP-94251 mass culture	der(9)t(9;17)(p10;q10), der(10)t(p15-15;?)
CP-94251 clone #2 (hTERT+)	der(9)t(9;17)(p10;q10)
CP-94251 clone #11 (hTERT+)	der(9)t(9;17)(p10;q10)
CP-18821 mass culture	der(1)t(1;22)(p?36;q?13), der(17)t(17;20)(p11;?)
CP-18821 clone #10 (hTERT+)	der(1)t(1;22)(p?36;q?13), der(10)t(10;15)p11;q11, der(17)t(17;20)(p11;?), inv dup(22) (pterq13)
CP-18821 clone #12 (hTERT+)	der(1)t(1;22)(p?36;q?13), der(3)(3;10)(?q11;?p26), dup(6)(?q), der(17)t(17;20)(p11;?), der(21)t(3;21)(q11.2-12;p12-13), inv dup(22) (pterq13)

Chapter 5: Therapeutics

Introduction

For the past two decades, the incidence of esophageal adenocarcinoma has been increasing more rapidly than that of any other cancer in the United States (Blot,1993). Most of these cancers arise in people with Barrett's esophagus, a condition that develops as a complication of chronic gastroesophageal reflux in which the normal squamous epithelium of the esophagus has been replaced by metaplastic columnar epithelium. Until recently, esophageal adenocarcinomas were almost uniformly fatal, with a five-year survival of only 7% (Miller, 1973-89). Current regimens of cancer chemotherapy using 5-fluorouracil (5-FU) and/or cisplatin (and in combination with radiation) have not led consistently to long-term survival (Whittington, 1990; Urba, 1992; Walsh, 1996). However, recent improvements in endoscopic surveillance have led to the early identification of increasing numbers of patients with preneoplastic and genetic changes in Barrett's epithelium or early intramucosal esophageal adenocarcinoma (Reid, 1987; Reid, 1992; Levine, 1993). Esophagectomy is presently the standard treatment for patients who develop an intramucosal Barrett's adenocarcinoma, but esophagectomy has substantial morbidity and mortality that makes it less than ideal as a treatment for cancer and precludes its use in routine cancer prevention (Muller,1990). Thus, there is a need for innovative therapies that can prevent the progression to cancer in Barrett's esophagus, as well as non-invasive and cost-effective therapies that are effective for the treatment of intramucosal esophageal adenocarcinoma.

Several groups of investigators have attempted to treat high-grade dysplasia or early adenocarcinoma in Barrett's esophagus using local ablative techniques, such as laser therapy, photodynamic therapy, or multipolar electrocoagulation. Each of these techniques has been reported to result in partial regression of Barrett's epithelium. However, the available evidence suggests that the success of these techniques may be limited, with local failure rates for early esophageal adenocarcinomas as high as 78% (Sibille, 1995). Attempts to use balloons to eliminate shadowing that may contribute to local photodynamic treatment failure have been only partially successful, but these experiments have demonstrated the feasibility of using balloons to distend the esophagus for local therapy *in vivo*. It is

possible that balloons may be used to physically isolate a segment of Barrett's esophagus for local instillation of therapeutic agents at concentrations above those that are tolerated systemically

p53 is a tumor-suppressor gene, located on chromosome 17p, that is inactivated in approximately 50% of human cancers and 90% of Barrett's adenocarcinomas (Hollstein,1991; Neshat,1994). p53 is required for at least two cell cycle checkpoints that prevent the propagation of genetically aberrant clones of cells by causing cell cycle arrest after genotoxic injury. A p53-dependent G1 checkpoint responds to DNA strand breaks by causing arrest in G1 and, under some conditions, activating a program of apoptosis, thereby preventing replication of the damaged DNA (Kastan,1992; Lowe,1993). Inactivation of the p53-dependent G1 checkpoint permits replication of the damaged DNA, which then leads to cell cycle arrest at a G2 checkpoint with a 4N DNA content. Most recently, p53 has been shown to prevent the polyploidization of cells exposed to anti-microtubule agents such as colcemid and nocodazole (Cross, 1995; Lanni, 1998).

p53 is inactivated by mutation of one allele and loss of heterozygosity involving the other (17p LOH) in approximately 90% of Barrett's cancers (Neshat, 1994b; Blount, 1994). Inactivation of p53 occurs in diploid, premalignant epithelial cells as an early event in neoplastic progression in Barrett's esophagus, prior to the development of aneuploidy and cancer (Blount,1994). This inactivation is coincident with the development of increased 4N (G2 or tetraploid) cell populations that are genetically unstable and predisposed to progression to aneuploidy and cancer in patients with Barrett's esophagus (Reid, 1992; Galipeau,1996). The data suggest that experimental therapeutic agents that selectively target p53-/- cells in premalignant Barrett's epithelium may be useful in preventing the development of cancer in this condition.

p53-/- cells are relatively resistant to many chemotherapeutic agents because they lack the normal cell cycle checkpoint and apoptotic responses to these compounds. However, there have been numerous reports of agents that appear to preferentially target cells lacking wild-type p53 function (Fan,1995; Hawkins,1996; Powell,1995; Russell,1995). The National Cancer Institute (NCI) has implemented an

investigational, *in vitro*, disease-oriented, drug discovery screen to identify substances that may have chemotherapeutic value (Monks, 1991). The COMPARE algorithm, which is a computer algorithm used to analyze the effects of individual compounds on different cell lines used by the NCI in drug screening, has been useful in identifying new antimitotic agents (Monks,1991). Recently, a p53 COMPARE study generated a list of 54 "p53-inverse compounds" (a subset of the over 30,000 compounds in the NCI screen) that appear to have preferential activity against cancer cell lines that have p53 mutations (Weinstein, 1997; O'Connor, 1997). This list of "p53-inverse compounds" should be viewed as a "hypothesis generator" suggesting that the compounds may have an increased probability of activity against p53-/- cells, but for which experimental proof will be necessary. We proposed to use the Barrett's epithelial cell strains in a drug screen that might allow the identification of p53-inverse compounds as potential chemotherapeutic agents. One of our principle goals was to establish the feasibility of using these "p53-inverse compounds" to selectively target the p53-/- epithelial cell lines that we have derived from patients with Barrett's esophagus. If selective activity could be found, this would support the contention that this strategy might be used to kill or inhibit the growth of the premalignant p53-/- Barrett's epithelial cells, while allowing genetically normal cells to regrow.

As discussed above, p53's checkpoint role in regulating the cell cycle in response to DNA damage has been well-documented. The differential response to damage between p53+/+ and p53-/- cells may be utilized for therapeutic advantage because p53-/- cells are defective in a number of cell cycle checkpoints and may, therefore, be vulnerable to agents that act at the remaining functional p53-independent checkpoints. Several groups have shown that cells with mutant p53 are more sensitive to pairs of agents, in which one (e.g., radiation or cisplatin) induces DNA damage while the other (e.g., pentoxifylline or caffeine) inactivates the remaining checkpoint (G2 in these examples). Thus, combinations of radiation plus caffeine (Powell,1995), radiation plus pentoxifylline (Russell,1995), and cisplatin plus pentoxifylline (Fan,1995) have activity against p53 mutant cells presumably because cells with mutant p53 are unable to arrest at either the G1 or G2 checkpoints, continue cell cycle progression and eventually die due to irreparable genetic damage. In those instances in which

selective activity is found, future work could be directed towards establishing the mechanisms of action by studying cell cycle checkpoint and apoptotic responses. Because p53 inactivation almost always occurs as an early event in neoplastic progression in Barrett's esophagus, removal of p53 mutant cells in premalignant Barrett's epithelium may prevent the occurrence of cancer in this condition.

Another method of selectively targetting cells that lack p53 involves the use of an adenovirus that is defective in a protein that normally inactivates p53 during viral infection (Bischoff,1996). The mutant dl1520 adenovirus (renamed ONYX-015) contains a deletion and point mutation in the E1B region which prevents the expression of the E1B 55kDa protein (Barker,1987). The E1B 55kDa protein binds p53 in order to prevent apoptosis of the infected cell. If a cell does not contain functional p53 protein, the E1B kDa protein becomes superfluous and an E1B deficient adenovirus can still infect and replicate within the cell. However, if a cell contains intact p53 function, the mutant virus will not be able to replicate efficiently in the p53 competent cell because E1B kDa protein is not present to block p53 mediated apoptosis. ONYX-015 was shown to cause cytopathic effect in tumor cells containing inactivated p53 and caused regression in tumor xenografts in nude mice, suggesting an *in vivo* anti-tumoral effect (Bischoff, 1996). ONYX-015 function in killing human tumor cells has been shown to be augmented by the chemotherapeutic agents cisplatin and 5-fluorouracil (Heise, 1997). Interestingly, ONYX-015 was shown to kill wild-type p53 tumor cells, however, it was not clear whether the p53 pathway had been perturbed in another manner such as MDM2 upregulation. In all cases, the normal human cell types tested appeared to be less sensitive to ONYX-015 viral infection than tumor cells (Heise, 1997). Most recently, intravenous administration of ONYX-015 in immunodeficent mice containing tumor xenografts appeared to show antitumoral activity (Heise, 1999).

In the past, the lack of an animal model or well-characterized cell strains from premalignant Barrett's epithelium or intramucosal esophageal adenocarcinoma has made preclinical studies of interventions in Barrett's esophagus difficult. We have, however, isolated Barrett's epithelial cell strains from

endoscopic biopsies and shown that they contain the genetic abnormalities that lead to cancer *in vivo* (Palanca-Wessels, 1998). These genetic abnormalities may provide a means to target the precancerous cells, particularly if local therapy can be directed to the esophagus, thereby minimizing systemic toxicity. We propose that these cell strains may be used to investigate the efficacy of promising chemical and viral therapeutic agents *in vitro*. The following describes preliminary experiments investigating possible therapies using the Barrett's epithelial cell strains.

p53 Inverse Agents and Checkpoint Abrogators

As part of the National Cancer Institute's (NCI) ongoing drug screening program, the NCI COMPARE algorithm has been used to identify chemical compounds that may show efficacy toward tumors lacking p53 function (Weinstein, 1997; O'Connor, 1997). We, in collaboration with the Drs. Edward Sausville and Kenneth Paull of the NCI, further revised this list to 31 compounds by giving greater weight to compounds that appear to show preferential cytotoxic and cytostatic effects on p53 mutant *epithelial* cell lines (Table 5.1). We chose to start with bisacodyl since it was non-proprietary and had been used clinically as a carthartic agent, and therefore its toxicity *in vivo* was known. We also investigated the use of pentoxifylline, a G2 checkpoint inhibitor that was not on the p53 inverse compound list, as another p53 selective agent because it had been shown in the past as exhibiting preferential toxicity to p53-/- cells (Russell, 1995).

We treated p53-/- (CP-52731) and p53+/+ (KR-42421) Barrett's esophagus epithelial cells with either bisacodyl (a "p53 inverse compound") or pentoxifylline (a G2 checkpoint abrogator) in order to determine whether either of the compounds cause selective growth inhibition of p53-/- Barrett's epithelial cells. Asynchronous cell cultures were plated in 96 well plates and exposed to varying doses of either bisacodyl or pentoxifylline. 17 hours after drug addition, 1 μCi of tritiated thymidine was added to the cells in order to determine the proportion of cells that were able to enter S phase after exposure to drug. After 48 hours of drug exposure, the cells were harvested onto filters and the tritiated thymidine uptake was determined. Results are shown in the Figure 5.1. At the higher drug doses (155 μg/ml for bisacodyl and 1.2 mg/ml pentoxifylline), both bisacodyl and pentoxifylline

showed greater growth inhibitory effects on the p53-/- cell line (CP-52731) as compared to the p53+/+ cell line (KR-42421). The GI_{50} (drug concentration causing 50% growth inhibition) of bisacodyl in the NCI drug screening program for p53-/- cancer cell lines of *epithelial* origin was 3.1 μg/ml (8.7 μM) whereas for p53+/+ cancer lines the GI_{50} was 12.5 μg/ml (34.7 μM). The GI_{50} of bisacodyl for the p53-/- Barrett's cell strain was 155 ng/ml whereas the GI_{50} for the p53+/+ Barrett's cell strain was 1.55 μg/ml. Both these concentrations are less than the GI_{50} of bisacodyl for the NCI p53-/- epithelial cancer cell lines and suggest that bisacodyl may be a compound that should be investigated further as a possible therapeutic for Barrett's esophagus.

The use of high dose chemotherapeutics in the treatment of cancer has not been fully explored, mainly due to the systemic toxicity. However, Barrett's esophagus provides the unique opportunity for local instillation of drugs at high doses, which may be concentrations at which selective activity occurs for some of these agents. Finding doses tolerated locally and determining absorption rates will need to await later phases of animal studies in preparation for clinical Phase 1 trials. These preliminary results indicate that it may be possible to selectively target the p53-/- Barrett's epithelial cells present in premalignant Barrett's epithelium with p53 inverse agents.

E1B Mutant Adenovirus (ONYX-015)

Adenoviral infection of Barrett's esophagus cells in vitro requires high viral titer

Previous work had shown that tumor cell lines lacking p53 function were sensitive to adenovirus ONYX-015 infection whereas normal human cells were not (Bischoff, 1996). We wanted to determine whether our premalignant p53-/- Barrett's esophagus cell strains were equally permissive for ONYX-015 infection and replication. We began our experiments using the three Barrett's cell strains known to contain p53 mutations (CP-52731, CP-94251, and CP-18821). As a positive control, we used the colon carcinoma cell line HCT-116 that has been shown to be efficiently infected and killed by ONYX-015 (Heise, 1997).

CP-52731 and HCT-116 cells were infected with both wild-type and ONYX-015 adenovirus at multiplicity of infection (MOI) 0.1, 1.0, and 10. After greater than 90% of cells in wild-type adenovirus infected wells showed cytopathic effect (CPE) visually, cells were harvested and stained with the viability dyes fluorescein diacetate (FDA) and propidium iodide (PI) prior to analysis on the flow cytometer. Figure 5.1 shows the typical CPE of adenovirus, characterized by rounding up and detachment of cells from the plate. Viability was determined by the differential staining of live (FDA positive, green fluorescence) and dead (PI positive, red fluorescence). Results for CP-52731 at 28 days post-infection are shown in Table 5.2. Wild-type adenovirus caused loss of cell viability at all MOIs tested and at MOI 10 left only 0.2% viable cells relative to control. A reduction in viability in ONYX-015 infected wells was seen only at MOI 10 where there was 81.9% viability of cells relative to control. HCT-116 cells showed considerable CPE as early as 3 days after infection in wells infected at MOI 1.0 and 10 with either wild-type or ONYX-015 adenovirus (data not shown). By 10 days post-infection, HCT-116 cells infected by either virus at all MOIs showed nearly 100% CPE. CP-52731 appeared to be more resistant to infection by both the wild-type and ONYX-015 virus compared to the HCT116 cells. CPE could not be detected visually until 8 to 10 days post-infection in Barrett's cells infected with wild-type virus at MOI 10. CPE was delayed in CP-52731 compared to HCT-116 cells, taking 28 days to achieve 90% CPE in cultures infected with wild-type adenovirus. In CP-52731, no significant CPE was evident visually in ONYX-015 infected wells at the time the cells were harvested for the viability assay.

Because it appeared that even wild-type adenovirus had difficulty lysing the Barrett's cells, we investigated whether prior treament of Barrett's esophagus cells with mucolytics such as N-acetylcysteine (NAC) and dithiothreitol (DTT) could improve the infectivity of adenovirus. Like the gastrointestinal cells they resemble, Barrett's esophagus epithelial cells may produce mucus which can hinder the attachment and/or entry of adenovirus. Use of mucolytics has been shown to improve retroviral infection in the gastrointestinal tract (Jacomino, 1997; Sandberg, 1994). The effect of a two minute NAC rinse prior to viral infection was assessed in CP-94251 cells (Table 5.3). Rinsing cells with NAC prior to adenoviral infection did not appear to enhance the degree of CPE when compared

to rinsing cells with phosphate-buffered saline (PBS). Rinsing with NAC actually appeared to increase rather than decrease the percent of viable cells in the infected cultures.

We performed another experiment using another Barrett's cell strain, CP-18821, in order to confirm the previous results with NAC and to also test the effect of another mucolytic agent DTT. We rinsed cells with PBS, NAC, or DTT for two minutes prior to viral infection. The results are shown in Table 5.4. Rinsing with NAC or DTT did not significantly decrease the percent viability compared to rinsing with PBS. Thus, mucolytics do not appear enhance the ability of adenovirus to kill Barrett's cells *in vitro*.

Enhancement of adenoviral infectivity of Barrett's cells in vitro by co-incubation with lipofectamine

At this point in our studies, it was not clear whether the adenovirus had difficulty entering the Barrett's cells (perhaps due to virus receptor deficiency) or replicating within the Barrett's cells after viral entry. We performed a series of experiments to improve viral killing by increasing the viral titer up to MOI 80 and pre-incubating the virus with lipofectamine prior to infection. Pre-incubation of adenovirus with cationic lipids had been shown to increase the efficiency of adenoviral uptake in cells (Fasbender, 1997). There have also been precedents in the use of cationic liposome complexes in clinical gene therapy (Caplen, 1995; Porteous, 1997).

Our initial experiments indicated that preincubation of adenovirus with lipofectamine for at least 15 minutes prior to infection increased the CPE of ONYX-015. Table 5.5 shows the results of infection of CP-52731 cells at MOI 10 using lipofectamine at 750 ng or 1500 ng of lipofectamine per 9×10^9 plaque forming units (pfu). At 1500 ng of lipofectamine, the percent of viable cells was reduced a further 10% compared to the cells infected with ONYX-015 adenovirus alone.

Effect of Barrett's cell culture confluency at infection on adenoviral cytopathicity

Some investigators have reported that cells in S phase were more susceptible to productive infection by E1B 55kD mutant virus than cells in other phases of the cell cycle (Goodrum, 1997). We tested

whether infecting cells at plated at lower confluency would increase the degree of CPE observed. We hypothesized that plating cells at lower density would ensure that contact inhibition would be minimal and the proliferative index would be high. In the previous experiments, cells were at 70% confluency at the time of infection. We plated CP-94251 cells at 25%, 50% and 75% confluency then infected them with adenovirus preincubated with lipofectamine at 1500 ng per 9 x 10^9 pfu at MOI 10. Table 5.6 shows the results of infection of CP-94251 cells at varying cell density. Cell density at the time of infection did not appear to significantly affect CPE. However, plating at 50% confluency seemed to give the best results. Therefore, we performed the remainder of the experiments using 50% confluency at the time of infection.

Because we were concerned that measuring the percentage of live cells at late times after infection underestimated cell death (dead cells could lyse and not be detected by the flow assay), in the experiment described above, we modified the protocol to allow determination of relative cell numbers. By adding a known number of chicken erythrocyte nuclei (CEN) into each sample prior to running on the flow cytometer, the relative cell number between samples could be quantitated. Table 5.7 shows the relative number of live cells after infection of CP-94251 with virus and lipofectamine. Infection with ONYX-015 preincubated with lipofectamine reduced the number of live cells to 50% of control.

Increased viral titer coupled with lipofectamine co-incubation enhances adenoviral killing of Barrett's cells in vitro

Since incubation of virus with lipofectamine enhances CPE, this suggested that viral entry may be inefficient. If so, increasing viral titer could improve viral killing. We tested whether CPE could be improved by increasing viral titer with and without preincubation with lipofectamine. Previous experiments were performed using a maximum MOI of 10. We compared the CPE at MOI 10, 20, 40, and 80 with and without preincubation with 1500 ng of lipofectamine. Results examining both percent viability (Table 5.8) and cell number (Table 5.9) are shown. Increasing viral titer enhanced the degree of CPE. Preincubation with lipofectamine augmented CPE even more, but the effect

appeared to plateau at MOI 20. A second experiment examining the effect of increased viral titer and preincubation with lipofectamine confirmed these results (data not shown).

Esophageal squamous cells and p53 wild-type Barrett's cell strain are sensitive to ONYX-015 infection in vitro

We performed experiments using cultured squamous esophageal epithelial cells because these are the cells normally present in the esophagus and serve as a clinically relevant control cell type. We infected esophageal squamous cultures with adenovirus with (Table 5.11) and without lipofectamine (Table 5.12) preincubation. The normal squamous cells appear to be much more sensitive to ONYX-015 than the Barrett's esophageal cells *in vitro*. Even without lipofectamine-preincubation, the ONYX-015 virus caused a more dramatic reduction in viability and cell number than the wild-type virus. At MOI 0.1, viability in ONYX-015 infected cells decreased to 6.7% of control level whereas viability with wild-type virus was decreased to 20.6% of control at 11 days post-infection. At an MOI less than 1, this indicated that adenoviral replication may be occuring. We repeated the experiment using cells derived from biopsies of a different patient and found the same results (Table 5.13). This suggested that in esophageal squamous epithelial cells, the presence of wild-type p53 gene sequence is not enough to prevent the cytopathic effect and viral replication of ONYX-015 adenovirus. Other studies have suggested that the p53 pathway may be inactivated in stratified squamous epithelial cells (Flatt, 1998).

KR-42421, the Barrett's cell strain not containing 17p LOH or p53 mutation, was more sensitive to ONYX-015 infection than the other three Barrett's strains tested (Table 5.14). At MOI 10, cell viability was reduced to only 2% of the control culture even without preincubation of ONYX-015 adenovirus with lipofectamine. The p53 pathway in this cell strain, however, may be inactivated since we have observed that there was a lack of G1 arrest in response to radiation (Palanca-Wessels, data not shown). Previous work has shown that cancer cell lines containing wild-type p53 gene sequence may also occasionally be permissive for ONYX-015 infection and lysis suggesting that

other factors in addition to p53 genotype may determine the host range of ONYX-015 (Heise, 1997; Rothmann, 1998).

Ex vivo adenoviral infection of esophageal biopsies

Our preliminary *ex vivo* experiments appear to support the results from the *in vitro* experiments. H&E stained ONYX 015-infected and control slides were examined by two pathologists who were blinded to the treatment status of the tissue. Representative sections examined are shown in Figure 5.4. Both pathologists correctly identified the infected squamous epithelial biopsy. The untreated squamous biopsy exhibited normal architecture and cell morphology. The treated biopsy contained epithelial cells that were described as undergoing "severe acantholysis". One pathologist also observed pyknotic changes in the detaching epithelial cells. Both pathologists found it difficult to distinguish any difference between the uninfected and infected Barrett's esophagus biopsies. One pathologist found no "global difference" in either the morphology or amount of cell death present on the slides. The other pathologist correctly identified the infected Barrett's esophagus sample but described the difference as being very subtle. This same pathologist was able to identify the treated biopsy in another pair of treated and untreated biopsies. He was also able to identify squamous biopsies as infected in two other separate samples however the controls for these samples were lost in histopathology processing and could not be examined in comparison.

Discussion

The National Cancer Institute has implemented a screening program utilizing a number of cancer cell lines in order to identify promising chemotherapeutic agents (Monks, 1991). We describe the feasibility of testing of promising cancer therapeutics using the Barrett's cell strains. We studied whether a differential sensitivity to bisacodyl (a p53 inverse agent) or pentoxifylline (a G2 checkpoint abrogator) exists between p53-/- and p53+/+ Barrett's cells. Our results indicated that there may be enhanced cytotoxicity in Barrett's cells lacking p53 to both these agents. We also performed experiments to investigate the possible selective cytopathicity of the E1B 22kDa deficient adenovirus ONYX-015 for cells lacking p53 function. Our results indicated that use of high viral titer and

preincubation of virus particles with lipofectamine enhanced the CPE of ONYX-015 in Barrett's epithelial cells *in vitro*. We found, however, that normal esophageal squamous cells *in vitro* were very sensitive to ONYX-015; the data suggested that although the cells contained functional p53, ONYX-015 was able to replicate and kill the cells.

Some of the results from our experiments using ONYX-015 were unexpected. First, the Barrett's esophagus cell strains containing mutant p53 appeared to be relatively resistant to ONYX-015 compared to wild-type adenovirus, whereas the Barrett's cell strain containing wild-type p53 was more sensitive to ONYX-015. In previous studies, ONYX-015 appeared to effectively lyse and kill cells containing mutant p53 (Bischoff, 1996; Heise, 1997). Some recent studies, however, have shown that ONYX-015 viral replication is impaired in some cells lacking p53 and that p53 status may not be the only determinant of ONYX-015 susceptibilty (Rothmann, 1998; Harada, 1999). The Barrett's cell strains may contain a cellular factor that makes ONYX-015 replication inefficient; alternatively, the mutant p53 allele present in the Barrett's cell strains may retain some functionality. Another study suggested that although adenovirus may replicate in cells lacking p53, CPE may not be obvious because functional p53 is required for death or detachment of cells from the cell culture surface; CPE was delayed in p53 mutant cells infected with wild-type adenovirus (Hall, 1998). We had also observed a delay in CPE in the Barrett's cell strains compared to the HCT-116 colon carcinoma cells we used as a positive control. Viral replication may have been occuring, but the assays we used in our study may not have detected this. Our result showing heightened sensitivity to ONYX-015 infection in the p53 wild-type Barrett's cells may be due to inactivation of p53 by some other method such as overexpression of MDM-2. Other studies have also shown that p53 wild-type cancer cells are also susceptible to ONYX-015 cytopathicity (Heise, 1997; Rothmann, 1998; Harada, 1999).

A second unexpected observation in our study was the high sensitivity of the normal squamous esophageal cells to the ONYX-015 virus. Previous studies had shown that the normal cell types tested were relatively resistant to ONYX-015 CPE (Heise, 1997). Other studies, however, have shown that

ONYX-015 can replicate in normal human fibroblasts, neonatal kidney cells and mammary epithelial cells (Harada, 1999; Rothmann, 1998). Some data suggest that normal human keratinocytes contain an attenuated G1 arrest *in vitro* (Flatt, 1998) when treated with either γ-radiation or adriamycin, implying that although p53 is present, its function may be somewhat reduced in this particular cell type; in the same study, p53 protein accumulation after treatment was found to be less in keratinocytes when compared to normal fibroblasts. It may be that esophageal squamous epithelial cells do not contain fully functional p53 and that this may contribute to their sensitivity to ONYX-015.

The *in vitro* data using cultured esophageal squamous cells would suggest that use of ONYX-015 *in vivo* might cause side effects due to toxicity to normal esophageal squamous tissue. Preliminary clinical Phase 1 trials in head and neck cancer patients, however, seem to indicate that normal tissue adjacent to sites of ONYX-015 injection are not damaged whereas the p53-mutant tumors show CPE and necrosis (Kirn, 1998). Similarly, damage to normal esophageal tissue in preliminary studies in Barrett's esophagus patients appears to be minimal (Dr. Brian J. Reid, unpublished data). There are a number of reasons why the results from an *in vitro* cell system may differ from results *in vivo* and yet still be valid. Cell cultures are necessarily an artificial system, used because the conditions are easily manipulable. *In vivo*, the local environment is composed of many interacting cell types. The cells are bathed in a sea of growth factors and cell signalling molecules that may not be present *in vitro* where cells are usually grown in monolayer and are isolated from the cellular interactions that naturally occur *in vivo*. One possible explanation for the the sensitivity of the esophageal squamous cells to ONYX-015 *in vitro* may be that the cells are grown in monolayer, whereas *in vivo* the cells are grown in a stratified manner with more differentiated squames sloughing off naturally. The squamous layers which contain non-dividing differentiated squames may prevent the penetration of ONYX-015 to the basal layer containing stem cells, thus protecting this population of cells from viral infection. In this scenario, although the superficial layer of cells is infected and lysed, these cells slough off and the unaffected basal stem cells remain viable, proliferate, and re-epithelialize the esophagus. Barrett's epithelial cells at the metaplastic stage form a simple columnar epithelium (a single layer of cells) *in*

vivo and therefore testing ONYX-015 on Barrett's epithelial cells in monolayer culture may be sufficient. On the other hand, testing ONYX-015 on normal esophageal cells in a monolayer culture may have produced a contradictory result because the normal tissue architecture *in vivo* may confer a protective advantage to the cells. The experiments using an *ex vivo* technique, using squamous biopsies, were meant to preserve the tissue architecture. One problem, however, was that we did not try to optimize the *ex vivo* growth conditions of the biopsies. Therefore, tissue viability may not have been optimal and parts of the tissue may have become necrotic, as judged by the poor histology of some of the biopsies.

There are, however, other *in vitro* cell culture systems that more closely mimic the *in vivo* environment. Organotypic cultures attempt to recreate the cellular interactions that may take place in an organism by, for example, growing epithelial cells on stromal cells. In one system, normal esophageal squamous cells are plated on fibroblasts derived from esophageal tissue in transwell plates (Oda, 1997). When stained with hematoxylin and eosin, the architecture of the organotypic culture closely mimics the tissue *in vivo*. Differentiation of the esophageal squamous cells can be seen histologically as one examines the cells closest to the fibroblast layer and compares their morphology to the more superficial cells. Similar type raft cultures have been used to study human papilloma virus immortalized foreskin keratinocytes (Blanton, 1991; Merrick, 1992). Testing the ONYX-015 virus on esophageal squamous cells grown in this manner may give results that more closely match the results found *in vivo*. The development of an organotypic system utilizing the Barrett's cell strains is an attractive possibility since normal squamous esophageal cells can be grown in this tissue culture method.

There are other cell culture systems that attempt to mimic the three dimensional growth of tumors. Cells grown in multicellular spheroids simulate the microenvironments that exist in a solid tumor before angiogenesis occurs (Sutherland, 1988; Schwachofer, 1990; Durand, 1994). For example, the cells in the interior of a large tumor are more nutrient and oxygen deprived than the cells at the leading edge of the neoplasm. Cells growing in different microenvironments are likely expressing

different proteins and may behave in different ways when exposed to a chemotherapeutic agent. In a normal monolayer culture, all cells are equally exposed to nutrients and oxygen. In multicellular spheroids, cells in the interior of the spheroid are less exposed to the culture medium and are growing under nutrient deprived and hypoxic conditions as compared to the cells on the surface, thus mimicking the *in vivo* gradient found in a solid tumor. Another system that recreates a three-dimensional tissue environment but which avoids the microenvironments present in the spheroid model, and may be utilized in future experiments involving the Barrett's cell strains, is the CultiSpher™ system. In this cell culture technique, cells are seeded on macroporous gelatin beads (CultiSphers™) that are constantly agitated and bathed in cell culture medium. The cells seeded in the interior of the beads do not appear to show slower proliferation or decreased viability compared to cells present on the surface. Cells seeded in CultiSphers™ are more resistant to chemotherapeutic drugs such as radiation and cisplatin than cells grown in monolayer (Rasey, 1996; Maurer, 1999). With the collaboration of Dr. Marilyn Cornwell, we tested whether Barrett's cells would be able to be grown in the CultiSpher™ system. We found that the Barrett's epithelial cells were able to attach to the beads and maintained a normal cell cycle profile (unpublished observations). Thus, growing Barrett's cells on CultiSphers™ may be feasible and represents another approach to try to re-create what is seen *in vivo* and make *in vitro* screening of therapeutic agents more reliable.

Although there appear to be many drawbacks to using an *in vitro* system to screen for therapeutics, there are many advantages as well. Using cell cultures as a primary screen for possible therapeutic agents is more cost-effective than using animal models. Furthermore, some diseases, such as Barrett's esophagus, are not represented by a good animal model and testing in humans is impossible without preliminary data to show that a particular agent is a good candidate for therapy. Monolayer cultures, as discussed above, have drawbacks compared to more organotypic systems. However, use of organotypic systems and animal models is labor-intensive and costly which preclude their use in large-scale screening studies such as the National Cancer Institute's drug screening program. Monolayer cultures are more practical for initial screening purposes. In further support of the use of

in vitro cultures, a recent study reported that many of the gene expression differences found *in vivo* between normal and tumor-derived tissue persist *in vitro* in cancer cell lines, although one must be cautious in extrapolating the findings *in vitro* to the *in vivo* condition (Zhang, 1997).

In conclusion, while we observed promising results in preliminary experiments using bisacodyl and pentoxifylline in targetting p53-/- Barrett's cells, our experiments using ONYX-015 adenovirus suggested that esophageal squamous cells would be more sensitive to infection than the Barrett's epithelium and that this may cause side effects in patients. However, this does not appear to be the case based on the preliminary results of clinical studies. Using the cell strains for screening of possible therapeutics still has merit, however. As discussed above, there are other culture systems which may be utilized that can better mimic the *in vivo* environment and may be used for preliminary screening of promising therapeutic agents. Furthermore, with the establishment of immortal, hTERT transduced Barrett's cell cultures (Chapter 4), neoplastic progression (and tumor formation) may be able to be reproduced in an organotypic system that is not limited by cell culture lifespan or cell number. Use of an organotypic system recreating tumor progression *in vitro* may lead to the development of therapeutics that prevent or halt the formation of cancer.

Methodology

Cell culture maintenance

Barrett's cell cultures were fed three times a week with MCDB growth medium containing growth supplements as previously described (Chapter 1; Palanca-Wessels, 1998) Esophageal squamous cultures were established from endsocopic biopsies and fed KBM plus BEGM SingleQuots™ (Clonetics, San Diego, CA) as described (Chapter 1). E6/E7 transformation was performed as previously described (Farin, 1995).

Assays of drug effect

Cells were plated into 96 well plates 24 hours prior to either bisacodyl (Sigma, St. Louis, MO) or pentoxifylline (Sigma) exposure. After drug addition, cells were returned to culture for 48 hours. For

viability and cell cycle analysis, the cells were stained with 7-amino-actinomycin D (Calbiochem, La Jolla, CA) dye for 30 minutes at room temperature, and then stained with DAPI (4',-diamidino-2-phenylindole). The cells were harvested from the wells just prior to being run on the flow cytometer. For analysis of growth, 1 μCi tritiated thymidine (Dupont NEN Products, Boston, MA) was added to each well within 24 hours of drug exposure. After 48 hours of drug exposure, the cells were harvested onto filters and the tritiated thymidine uptake determined.

In vitro adenoviral infection

Cells were plated in 24 well plates the day prior to infection in order to achieve the desired confluency. If using lipofectamine, the virus was incubated with the appropriate amount of lipofectamine (Gibco, Grand Island, NY) with 9×10^9 plaque forming units for 15 to 30 minutes prior to infection. If using mucolytics, the media was removed from the plates and the cells were rinsed for 2 minutes in either N-acetylcysteine (Sigma) or dithiothreitol (Sigma). Virus or virus-lipofectamine complexes was added to the cells and incubated for 3 hours with occasional rocking of the plate. The inoculum was removed and cells were fed with infection media (regular growth media containing only 2% fetal bovine serum). Cells were fed as usual until >90% CPE was observed in wild-type adenovirus infected wells or until designated timepoint.

Ex vivo adenoviral infection

Endoscopic biopsies were rinsed with 2X antimycotic solution (10 μg/ml amphotericin B and 180 units penicllin/180 μg streptomycin per ml in sterile PBS) prior to incubation with adenovirus at the appropriate MOI in culture media containing 10% serum. After *ex vivo* culture for two to four days, biopsies were removed from media and fixed in 10% buffered formalin. Fixed tissue was paraffin embedded and sections cut prior to H&E staining.

Flow cytometric viability and cell count assay

Both floating and adherent cells were harvested from the cultures as usual. The cell pellet was resuspended in staining mix which consisted of growth medium containing 5 μg/ml propidium iodide (PI), 0.5 μg/ml fluorescein diacetate (FDA), and 10 μM Hoechst 33342 (HO) and incubated for 5 to 15 minutes in the dark at room temperature. For cell counts, a known quantity of BioSure Controls chicken erythrocyte nuclei (CENs) (Riese Enterprises, Grass Valley, CA) was added to the cells in the staining mix. The cells were run on the flow cytometer, triggering on HO. Right angle scatter versus PI or forward scatter was used in order to be able to gate on CENs. The gain was adjusted so as to not "cut off" CENs in the PI log versus FDA log histogram. To determine cell counts, histograms were analyzed by gating on CENs and cells individually. The formuala used was the following: Total cell number = [number of cells run] X [total number of CENs added to sample ÷ number of CENs run].

Table 5.1. List of p53 inverse compounds ranked by cytotoxic effect on p53 mutant epithelial cells.

NSC #	Common name (if available)
277763-S	
614826-W	Bisacodyl
155693-P	6H-Pyrido[4,3-b]carbazolium, 9-methoxy-2,5,11-trimethyl-,iodine
93739-W	Aizen magenta
352299-R	2-Methylellipticinum methanesulfonate
325663-P	Saframycin A
671456-A	3-(4-Fluorophenyl)-3-(4-acetoxy-2-methylphenyl)phthalide
626874-S	
642947-O	
682769-X	
684439-O	
633001-C	Benzene,1, 1'-sulfonyl-bis(2-nitro-)
639364-T	2-N-Methyl-9-methylellipticinium acetate
681732-V	
645647-X	Benzamide riboside
169688-A	Coralyne, nor
679743-K	
681734-X	
98542-S	Acetopapaverine
633253-A	
674454-L	(Z)-3-Iodo-1,2-diphenyl-2-propen-1-one
627585-Q	
674130-J	4-(1-Acetyloxypropen-2-yl-)-2-methoxyphenylisobutyrat
72055-C	Sulfonium, dodecyldimethyl-iodide
678125-Z	
85791-J	Ethacrynic acid
632855-T	9-Chloro-2-methylellipticinium acetate, hydrate
678063-J	
661237-T	
676614-J	
634398-V	

Table 5.2 Adenoviral cytopathic effect in CP-52371 Barrett's epithelial cells
% live cells in total cell fraction relative to uninfected control culture 28 days post-infection [n=2 except for MOI 10 (n=1); mean ± standard deviation].

Viral Titer	Wild-type	ONYX-015
MOI 0.1	58.3 ± 18.6	112.7 ± 9.6
MOI 1.0	10.0 ± 2.0	105.4 ± 1.0
MOI 10	0.2	81.9

Table 5.3 Effect of N-acetylcysteine (NAC) on adenoviral cytopathic effect in CP-94251 Barrett's epithelial cells
% live cells in total cell fraction relative to uninfected control culture 19 days post-infection [n=1 for PBS; n=2 for NAC; mean ± standard deviation].

Viral Titer	PBS rinse		Viral Titer	NAC rinse	
	Wild-type	ONYX-015		Wild-type	ONYX-015
MOI 0.1	75.9	99.1	MOI 0.1	85.4 ± 9.9	104.8 ± 0.9
MOI 1.0	51.9	87.7	MOI 1.0	53.3 ± 0.1	89.4 ± 8.7
MOI 10	11.9	56	MOI 10	13.8 ± 5.2	74.4 ± 12.2

Table 5.4 Effect of N-acetylcysteine (NAC) and dithiothreitol(DTT) on adenoviral cytopathic effect in CP-18821Barrett's epithelial cells
% live cells in total cell fraction relative to control 20 days post-infection [n=3; mean ± standard deviation]. The mucolytic used prior to infection is indicated in parentheses.

Viral Titer	Wild-type	ONYX-015
MOI 10 (PBS)	8.4 ± 1.9	81.0 ± 8.5
MOI 10 (NAC)	7.1 ± 1.0	73.8 ± 8.6
MOI 10 (DTT)	19.4 ± 3.3	82.9 ± 1.5

Table 5.5 Effect of lipofectamine on adenoviral cytopathic effect in CP-52731 Barrett's epithelial cells
% live cells in total cell fraction relative to control 9 days post-infection [n=3; mean ± standard deviation]. The amount of lipofectamine per 9 x 109 plaque forming units is indicated. An MOI of 10 was used in this experiment.

	Wild-type	ONYX-015
No lipofectamine	82.6 ± 7.3	88.7 ± 1.3
Lipofectamine 750 ng	71.2 ± 6.4	81.1 ± 5.5
Lipofectamine 1500 ng	70.3 ± 3.3	73.9 ± 6.1

Table 5.6 Effect of cell culture confluency on adenoviral cytopathic effect in CP-94251 Barrett's epithelial cells (% live cells)

% live cells in total cell fraction relative to control 12 days post-infection [n=3; mean ± standard deviation]. Virus particles were pre-incubated with 1500 ng lipofectamine.

Culture confluency at infection	Wild-type	ONYX-015
25% confluency	60.9 ± 5.8	70.8 ± 2.9
50% confluency	52.5 ± 3.1	55.7 ± 4.4
75% confluency	45.7 ± 5.3	62.8 ± 7.8

Table 5.7. Effect of cell culture confluency on adenoviral cytopathic effect in CP-94251 Barrett's epithelial cells (number live cells)

Number of live cells relative to control 12 days post-infection [n=3; mean ± standard deviation]. Virus particles were pre-incubated with 1500 ng lipofectamine.

Culture confluency at infection	Wild-type	ONYX-015
25% confluency	22.3 ± 3.1	51.1 ± 5.3
50% confluency	20.2 ± 1.3	30.5 ± 8.2
75% confluency	23.4 ± 5.1	55.4 ± 14.0

Table 5.8. Effect of increased viral titer (with or without lipofectamine) on adenoviral cytopathic effect in CP-94251 Barrett's epithelial cells (% live cells)

% live cells in total cell fraction relative to control 11 days post-infection [n=3; mean ± standard error of the mean].

Viral Titer	*ONYX-015 No Lipofectamine*	*ONYX-015 +1500 ng Lipofectamine*
MOI 10	39.0 ± 1.4	26.5 ± 0.8
MOI 20	33.5 ± 1.4	18.9 ± 0.4
MOI 40	26.1 ± 1.4	19.3 ± 1.1
MOI 80	27.0 ± 1.3	15.6 ± 0.8

Table 5.9 Effect of increased viral titer (with or without lipofectamine) on adenoviral cytopathic effect in CP-94251 Barrett's epithelial cells (number of live cells)

Number of live cells relative to control 11 days post-infection [n=3; mean ± standard error of the mean].

Viral Titer	*ONYX-015 -Lipofectamine*	*ONYX-015 +Lipofectamine*
MOI 10	26.3 ± 1.8	13.1 ± 0.4
MOI 20	20.2 ± 1.1	7.5 ± 0.3
MOI 40	14.9 ± 0.8	10.3 ± 0.9
MOI 80	15.3 ± 1.4	6.5 ± 0.6

Table 5.10 Effect of adenovirus infection on esophageal squamous epithelial cells (VG)
% live cells in total cell fraction relative to control 9 days post-infection [n=3; mean ± standard error of the mean]. *L=virus particles pre-incubated with 1500 ng lipofectamine.

Viral Titer	*Wild-type*	*ONYX-015*
MOI 10	40.0 ± 2.9	6.2 ± 0.1
MOI 20	46.8 ± 5.6	3.8 ± 0.2
MOI 40	49.6 ± 3.6	5.8 ± 0.7
MOI L10*	30.6 ± 1.6	6.0 ± 0.2
MOI L20*	25.3 ± 0.9	4.9 ± 0.2

Table 5.11 Effect of adenovirus infection on esophageal squamous epithelial cells (VG) second experiment
% live cells in total cell fraction relative to control 7 days post-infection [n=3; mean ± standard error of the mean]. *L=virus particles pre-incubated with 1500 ng lipofectamine.

Viral Titer	*Wild-type*	*ONYX-015*
MOI 10	36.9 ± 6.8	3.9 ± 0.4
MOI 20	47.0 ± 16.2	8.7 ± 0.4
MOI 40	36.4 ± 3.6	4.5 ± 0.8
MOI L10*	45.0 ± 3.0	14.6 ± 8.7
MOI L20*	22.4 ± 3.8	4.3 ± 0.7

Table 5.12 Effect of reduced viral titer on adenoviral cytopathic effect in esophageal squamous epithelial cells (VG) without lipofectamine
VG SQE P5 cells 11 days post-infection: %live cells in total cell fraction relative to control 11 days post-infection [n=3; mean ± standard error of the mean]

Viral Titer	*Wild-type*	*ONYX 015*
MOI 0.1	20.6 ± 1.9	6.7 ± 0.3
MOI 1.0	9.4 ± 0.3	3.4 ± 0.2
MOI 10	7.5 ± 0.4	3.9 ± 0.4

Table 5.13. Effect of adenovirus infection on esophageal squamous epithelial cells (MB) without lipofectamine
% live cells in total cell fraction relative to control 10 days post-infection [n=3; mean ± standard error of the mean]

Viral Titer	*Wild-type*	*ONYX 015*
MOI 0.1	10.4 ± 0.5	42.4 ± 0.5
MOI 1.0	2.9 ± 0.3	1.2 ± 0.1
MOI 10	4.7 ± 0.5	1.0 ± 0.2
MOI 20	2.4 ± 0.2	1.1 ± 0.0
MOI 40	1.4 ± 0.2	1.0 ± 0.1

Table 5.14. Effect of adenoviral infection on p53+/+ Barrett's epithelial cells (KR-42421) % live cells in total cell fraction relative to control 11 days post-infection [n=3; mean ±standard error of the mean]

Viral Titer	*Wild-type*	*ONYX 015*
MOI 0.1	40.7 ± 7.2	113 ± 6.3
MOI 1.0	13.6 ± 1.7	57.3 ± 16.8
MOI 10	12.5 ± 1.5	2.0 ± 0.4
MOI 20	11.1 ± 0.9	0.8 ± 0.0
MOI 40	7.0 ± 0.6	0.8 ± 0.1

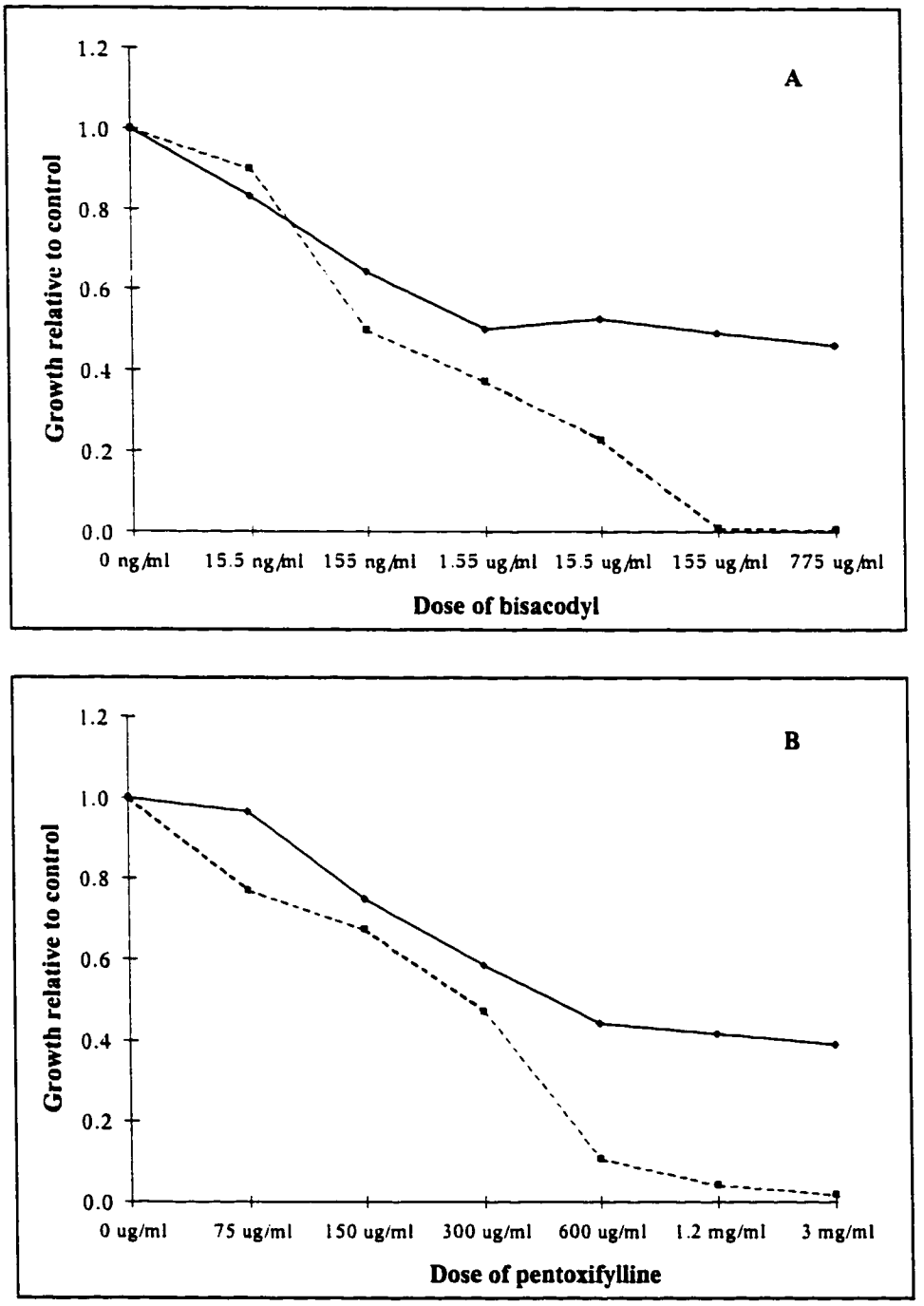


Figure 5.1 Effect of bisacodyl and pentoxifylline treatment on Barrett's epithelial cells. Cells were treated at varying doses of bisacodyl (*Panel A*) and pentoxifylline (*Panel B*). Tritiated thymidine uptake was assessed in the cell cultures after 48 hours of drug exposure. Results from the treatment of p53+/+ Barrett's cell strain KR-42421 (solid line) and p53-/- Barrett's cell strain CP-52731 (dashed line) are shown.

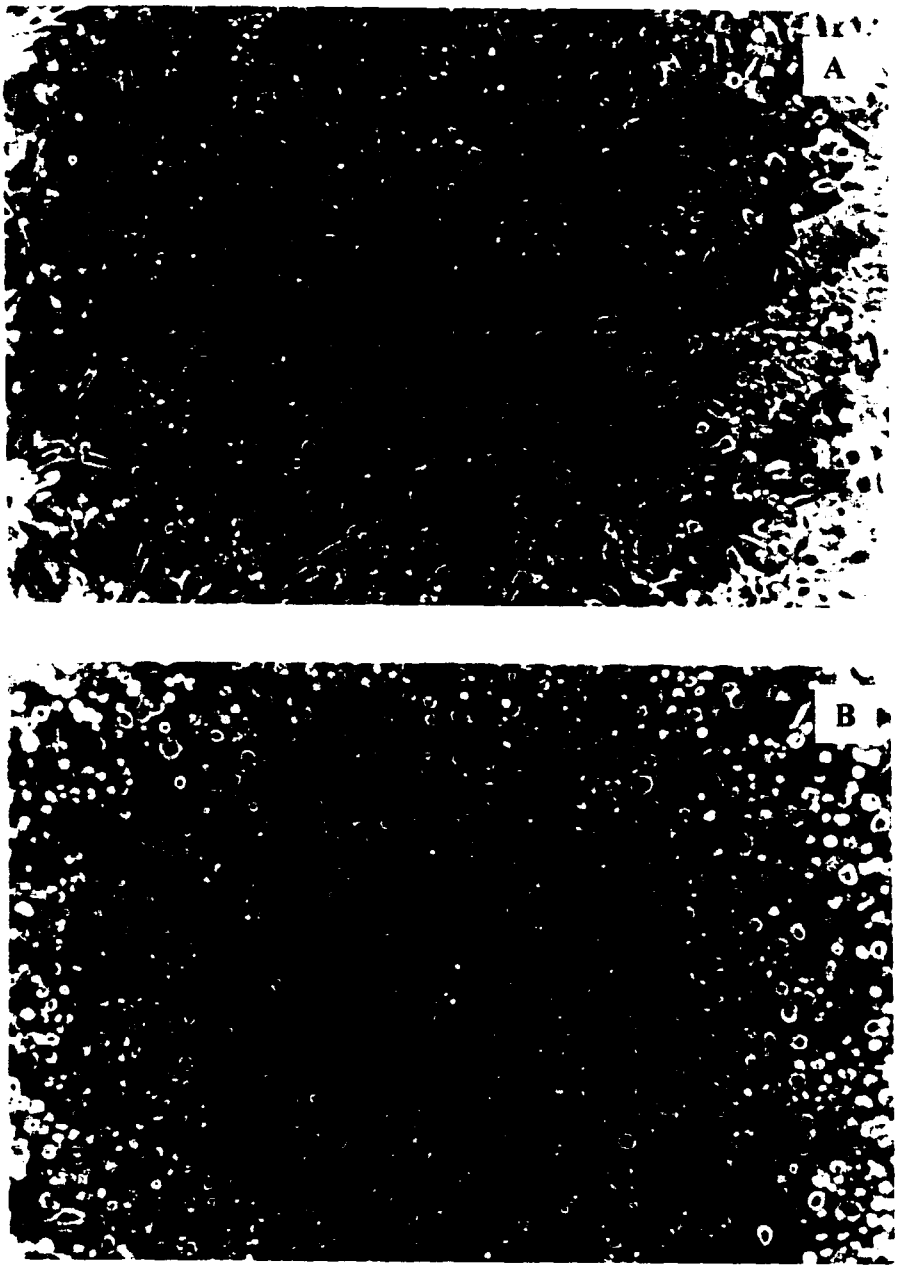


Figure 5.2 Cytopathic effect of adenovirus. Shown are Barrett's cell cultures *A*, not infected with virus and *B*, infected with wild-type adenovirus at MOI 10, fourteen days post-infection. The cytopathic effect is characterized by the rounding up and detachment of cells from the tissue culture plate.

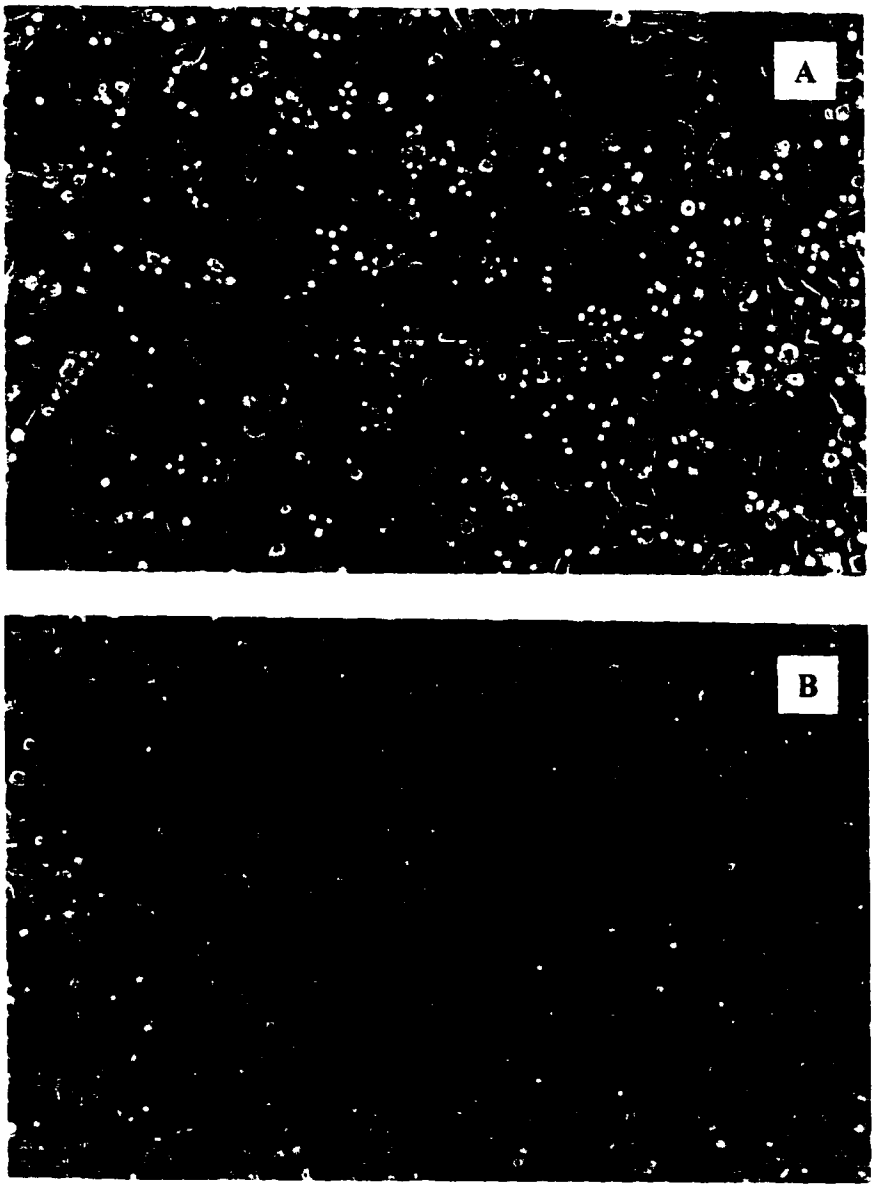


Figure 5.3 ONYX-015 infection of esophageal squamous cells. Shown are esophageal squamous cell cultures *A*, not infected with virus and *B*, infected with ONYX-015 adenovirus at MOI 10, six days post-infection. Cytopathic effect is evident in the ONYX-015 infected culture.

Figure 5.4 *Ex vivo* infection of esophageal biopsies. Shown above are hematoxylin and eosin stained sections of esophageal squamous biopsies *A*, not infected with virus and *B*, infected with ONYX-015 virus during *ex vivo* culture.

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