REVIEW

Stage matters: choosing relevant model systems to address hypotheses in diet and cancer chemoprevention research

Jennifer L. Fenton1,2,∗ and Norman G. Hord2

1Cancer Prevention Fellowship Program, Division of Cancer Prevention, National Cancer Institute, Bethesda, MD 20892, USA and 2Department of Food Science and Department of Human Nutrition, Michigan State University, East Lansing, MI 48824, USA

∗To whom correspondence and requests for reprints should be addressed at: Cancer Prevention Fellowship Program, NCI, Division of Cancer Prevention, 6130 Executive Boulevard, MSC 7361 Bethesda, MD 20892-7361, USA

Email: jeniferf@nci.nih.gov

Clinical evidence reveals that the efficacy of dietary factors to prevent cancer is probably stage-dependent. The ability to demonstrate stage-specific effects of dietary compounds on normal, preneoplastic and malignant cell models may provide insights into puzzling clinical results from cancer chemoprevention trials. The relevance of these models to the field of cancer prevention is immense and will undoubtedly facilitate the ability to discover which dietary factors are most effective at preventing cancer and which, if any, specific steps in neoplastic transformation render cells refractory to the effects of dietary compounds. There are illustrative examples where exposure of high-risk individuals to dietary chemopreventive agents increases rather than decreases cancer risk. While geneticists and clinical oncologists acknowledge the morphological continuum along which tumors develop in specific tissues, tumor cells, rather than normal and preneoplastic cells, continue to be the primary in vitro reductionist tool employed to elucidate mechanisms underlying disease progression and to investigate the potential utility of dietary as well as other chemopreventive agents. Currently, there are few relevant model systems to study the progression of neoplastic transformation, especially in epithelial cells. We highlight examples of model systems isolated from prostate, breast, endometrial and intestinal tissue, with special emphasis on a specific set of non-tumorigenic, conditionally immortal cell lines derived from C57/B16 mice [YAMC (Young Adult Mouse Colon cells; Apc+/+); ApcMin/−] cells and IMCE (Immorto-Min Colonic Epithelium cells; ApcMin/+; C3) cells) that have yielded important information on early events in colorectal neoplasia development. These cell lines are an illustrative example of how researchers can examine stage-dependent effects of specific dietary components on carcinogenesis. The utilization of cell culture systems modeling early, middle and late stages of tumorigenesis will yield important insights into mechanisms by which dietary components impact cancer progression.

Abbreviations: APC, adenomatous polyposis coli; ATRA, all-trans-retinoic acid; Caco-2, Caucasian colon adenocarcinoma-2; CEC, colon epithelial cell; CRC, colorectal cancer; ECM, extracellular matrix; FAP, familial adenomatous polyposis; IMCE, Immorto-Min colonic epithelium; YAMC, young adult mouse colon.

Introduction

Normal human epithelial cells, by definition, have a finite lifespan and do not undergo spontaneous immortalization. Cellular immortalization in humans (in vivo) typically requires, over the course of decades, mutational inactivation of critical cell proteins regulating cell cycle progression, apoptosis, cell–cell communication and senescence (1). Even so, important mechanistic insights into carcinogenesis have been gained using cell lines that display phenotypes relevant to malignant tumors. However, one might question whether these are the most relevant models to use for cancer prevention research, particularly when examining the ability of dietary factors to prohibit development of and alter progression of preneoplastic cells to cancer. While geneticists and clinical oncologists acknowledge the morphological continuum along which tumors develop in specific tissues (2), tumor cells continue to be the primary reductionist tool employed in vitro for chemoprevention research. That is, tumor cells, rather than normal and preneoplastic cells, are commonly employed to elucidate mechanisms underlying disease progression and to investigate the potential utility of dietary as well as other chemopreventive agents.

In reality, there is a deficiency of relevant model systems to study the progression of neoplastic transformation especially in epithelial cells. The commercial availability of normal human cell lines and conditionally immortal cell lines, however, has expanded the tools of the cancer scientist in this area. The relevance of these models to the field of cancer prevention is immense and will undoubtedly facilitate the ability to discover which dietary factors are most effective at preventing cancer and which, if any, specific steps in neoplastic transformation render cells refractory to the effects of dietary compounds. Consistent with the conclusions of the NCI Think Tank in Cancer Biology, understanding the determinants of the earliest detectable phenotypes in initiated cells and their tumor microenvironment is critical for the discovery of therapeutic targets in cancer (2). It is likely that the ability to demonstrate stage-specific effects of dietary compounds on not only malignant cells but on normal and preneoplastic cells may provide powerful insights into puzzling clinical results from early and recent cancer chemoprevention trials. The purpose of this commentary is to highlight relevant in vitro systems that can be utilized as tools for cancer prevention researchers to gain a better understanding of the stage-specific effects of purported dietary compounds on cancer chemoprevention.

It is estimated that 30–40% of cancers are directly linked to diet-related factors (3). Yet, understanding how specific dietary components impact cancer risk has eluded our understanding. This may partially be due to the dynamic and progressive nature of cancer characterized by the accumulation of mutations in cells that alter the cellular response to their environment. The Carotene and Retinol Efficacy Trial
(CARET) study is an excellent example of this phenomenon, where \( \beta \)-carotene supplementation in smokers actually increased lung cancer risk (RR = 1.16, 95% CI = 1.02–1.33; \( P < 0.02 \)) (4).

Researchers have developed breast, prostate, endometrial and intestinal tumor cell lines that serve as rudimentary models of these cancers, which will be reviewed in detail below. These models may provide for a better understanding of the ramifications of the stage-dependent effects of dietary compounds on neoplastic progression. Cell lines have proven useful in elucidating cell signaling pathways and genes involved in carcinogenesis. Weinberg et al. (5) demonstrated that primary human mammary epithelial cells could be rendered tumorigenic in nude mice by the introduction of SV40 large T antigen, the telomerase catalytic subunit, and an H-ras oncoprotein. When these cell model systems accurately reflect validated human biomarkers of risk, their utility is greatly enhanced. In contrast, most cells in culture are, by definition, immortal. As a result of cytogenetic abnormalities consistent with metastatic tissue, these models have limited utility for studies of dietary compounds in cancer prevention. For example, the commonly used human Caucasian colon adenocarcinoma (Caco-2) cell line is tumorigenic in nude mice, is polyploid and has numerous cytogenetic abnormalities. We will review several in vitro cell culture models currently available whose application to studying early events in cancer progression represents more relevant model systems than tumor cells alone. Additionally, we will propose that a specific set of non-tumorigenic, conditionally immortal cell lines derived from C57/BL6 mice might serve as an excellent illustrative example of an appropriate model system for cancer prevention. This model system has yielded important knowledge on early events in colorectal cancer (CRC) that may have application to human CRC carcinogenesis and the impact of diet and endogenous metabolic mediators on that process.

Why does stage matter for chemoprevention?

Recent illustrative examples that highlight null or negative data being generated from several recent cancer chemoprevention trials will be discussed below. These data are reminiscent of nutrients and putative chemopreventive agents that have been demonstrated to cause cancer (6). Dose and temporality are important potential explanations for the carcinogenic effect of nutrients in these cases. Investigators, largely owing to technological limitations in assessing preneoplastic lesions, are probably enrolling individuals with heterogeneous risk for developing cancers. Limited by detection technology, it is simplistic to assume that all individuals accrued into primary prevention trials are free of preneoplastic lesions. Having established this, it is rational to assume that current chemoprevention trials may be unknowingly accruing individuals with undetectable and perhaps heterogeneous preneoplastic lesions. Administering putative chemopreventive agents to individuals at heterogeneous risk is likely to yield unanticipated results. Since individuals cannot be assessed for preneoplastic stage or field defects (a region of genetically unstable, potentially precancerous cells), we may be observing the textbook definition of a confounder: cancer stage as an unidentified effect modifier. In the absence of morphological markers of preneoplastic stage, the only surrogate risk marker available are estimates of lifestyle exposures. We will illustrate this point by examining the stage-dependent effects of nutrients using associations between \( \beta \)-carotene consumption and lung cancer, folate intakes and CRC risk and soy consumption and breast cancer risk.

Strong evidence from over 30 case–control and cohort studies indicated that people who eat more foods rich in carotenoids, and carotenoids (\( \beta \)-carotene in particular), as well as those with higher blood \( \beta \)-carotene concentrations, have a lower risk of lung cancer than those who eat fewer such foods or have lower \( \beta \)-carotene concentrations (4,7). However, attempts to impute the preventive efficacy of fruit and vegetable-containing diets to one isomer of one carotenoid in large, controlled trials of \( \beta \)-carotene supplementation [e.g. Alpha-Tocopherol, Beta-Carotene (ATBC) Prevention Study and CARET trials] do not support the observed beneficial associations or a role for supplemental \( \beta \)-carotene in lung cancer prevention; instead, they provide striking evidence for adverse effects (i.e. excess lung cancer incidence and overall mortality) in smokers (4,7,8). It is unresolved whether these effects were due to unknown carcinogenic properties of \( \beta \)-carotene, undetectable preneoplastic lesions in smokers promoted by \( \beta \)-carotene, or some combination of the two. It is possible that \( \beta \)-carotene revealed those individuals expressing smoking-related preneoplastic lesions by promoting the progression of these lesions to clinically detectable lung cancer. It is noteworthy that light smokers did not experience the carcinogenic effects of \( \beta \)-carotene supplementation, indicating a possible carcinogen dose effect (9). Further supporting a possible dual effect of \( \beta \)-carotene supplementation, recent research showed that tobacco-related cancer risk was inversely associated with \( \beta \)-carotene intake in a dose-dependent fashion and was associated with risk among smokers (10). As such, \( \beta \)-carotene appears to be protective early, before cancer develops, but not in the context of premalignant/malignant lesions. Indeed, \( \beta \)-carotene causes the proliferation of pulmonary adenocarcinoma cells in culture (11). These results from the ATBC and CARET trials support the stage-dependent effects of \( \beta \)-carotene supplementation on lung cancer risk and highlight the necessity for relevant model systems for studying the effects of dietary compounds on cancer risk.

Similar to \( \beta \)-carotene, trials investigating folate supplementation and CRC risk provide an example of a nutrient whose effects on carcinogenesis also appears to be dependent on stage. The relationship between folate and CRC carcinogenesis is complex, while low-folate status early in life may enhance CRC carcinogenesis; excess folate intake may be harmful in certain situations (12). In animal models, folate supplementation is an effective chemopreventive agent if given before the establishment of early colorectal lesions. However, once a preneoplastic lesion is present, folate has been shown to enhance tumor growth (13–14). Further evidence from a randomized controlled trial demonstrated that folic acid supplementation among patients with resected adenoma enhanced the recurrence of multiple or larger adenomas (15). On the basis of these observations and the lack of ability to determine individuals in a population with precursor lesions, folic acid fortification policies warrant review. It is not currently known which types of precursor or preneoplastic lesions will regress or be promoted by folate supplementation. In fact, anti-folate therapy is common in cancer treatment. This ambiguity necessitates the identification of a model system to dissect the stage-specific effects of folate on CRC carcinogenesis.
Soy/isoflavone supplementation and breast cancer risk is another excellent example of risk modification by a dietary component that, in animal models, has been shown to be dependent upon form, dose and/or stage of life exposure (16). Genistein (the primary isoflavone in soybeans) has been found to both inhibit and promote mammary tumorigenesis as well as augment proliferation of cancer cells in vitro. It is probable that these conflicting findings are due to a dose effect of genistein on mammary cancer cell proliferation as low doses of genistein are mitogenic, but at high doses genistein is generally antiproliferative (17). This mitogenic effect is more consistent in estrogen receptor positive (ER+) cells but not in ER negative (ER−) mammary carcinoma cell lines (16,18).

Timing of consumption also appears to be crucial to understanding the differential effects of genistein (14). Epidemiological data and animal studies indicate that a life-time exposure, especially prepubertal, to soy/isoflavones is protective against developing breast cancer (19–20). However, some research shows no effect of genistein exposure in adult women on breast cancer risk (21) or no effect at all (22). Clearly, these multiple actions of soy/isoflavones are not conclusive and research continues actively in this area in an attempt to elucidate the dual effects of genistein. The available data currently lack convincing support to make public health recommendations concerning the consumption of soy and cancer risk particularly in ER+ breast cancer survivors.

These examples highlight the differential effect of dietary compounds on not only the stage of preneoplastic lesion but also the life stage timing, dose, and specific genetic differences in how cells may respond to specific dietary compounds. There are obvious limitations hindering the development of in vitro models of cells from specific life stages; mimicking dynamic changes in stromal and immune cell composition that occur during aging would be particularly challenging. However, the use of cells of various stages of differentiation (or, in the case of neoplastic cells, transformation state) may, in some cases, accurately reflect the differentiation or transformation state of cells within specific life stages. Further, they emphasize a critical need for researchers to utilize models of cancer progression that can address the response of cells in a specific stage of neoplastic growth to dietary compounds in addition to choosing the appropriate chemopreventive compound. With recently developed in vitro models available today, appropriateness of applying a compound to models that are metastatic and tumorigenic (i.e. Caco-2) is limited. Arguably, the resulting studies are, in fact, testing the efficacy of dietary compounds as cancer treatment agents rather than cancer chemopreventive agents.

Conducting cancer prevention research in vitro: a matter of stage

An ideal manner in which to study neoplastic transformation would be to isolate and culture a series of cells harboring from one to multiple mutations in different oncogenes, tumor suppressor genes and other cancer susceptibility genes relevant to carcinogenesis in a particular tissue. These observed phenotypic changes or ‘stages’ are categorized into phases coined ‘initiation’, ‘promotion’ and ‘progression’ and are pathologically regarded as the dysplasia or adenocarcinoma sequence (Table I provides a brief review of the differences observed in just three possible cell stages). In the absence of such cells, impractical using current technologies, we are left to develop more modest cell systems that mimic fewer genetic alterations and phenotypes of neoplastic cells than currently available tumor cell lines. Investigators acknowledge a need for these types of continuum model systems, and significant effort is being placed on the development of these model systems for many cancer types. Current in vitro model systems are limited by biological as well as logistical obstacles. Few systems make any attempt to account for the critical influence of the extracellular matrix (ECM) and the heterogeneous cell types surrounding epithelial cells.

Certainly, it is impossible to accurately mimic the specific environment and the influences of other cells in vivo. Nonetheless, it has proven almost impossible to dissect out the biological effects of dietary compounds on stage-specific cells without systematic reductionist approaches. Simply put, reductionism refers to a philosophical paradigm in which one attempts to explain complex phenomena using relatively simple principles; for example, using cells in culture to glean relevant information about neoplasia in a complex physiological context. However, these model systems, when applied to rigorous hypothesis-driven experiments, can yield important insights into the cell differentiation stage-dependent effects of dietary compounds. We will review several model systems currently available in the following discussion.

Organ culture

Optimally, cancer progression model design should reproduce the 3D organization, the community of heterotypic cell types and the differentiated function of any particular tissue. The vast majority of in vitro research has primarily been conducted using cells that are cultured as monolayers on tissue culture plastic or in soft agar assays, neither of which recapitulate the structural organization in vivo. Abundant research describes the manner in which the stromal microenvironment of in vivo tissue profoundly influences tumor progression (23). Therefore, 3D organ (and organotypic) culture is arguably the most relevant in vitro model system to study stage-dependent effects of dietary compounds on tumor progression. Because of the ethical, technical and financial constraints inherent in research using human tissues, the demand for human tissue considerably outweighs the supply. Therefore, researchers are developing animal organ cultures and

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3D organotypic cultures (both monotypic and multicellular) that may be useful for cancer chemoprevention research. Examples of this type of model can be gleaned from both prostate and mammary research in particular, as these are both normally glandular tissues.

Prostate tissue can be obtained from non-malignant and malignant human prostate surgical specimens to represent a continuum of cancer progression. Varani et al. (24) identified culture conditions for the successful maintenance of human prostate tissue for several days in organ culture and the histological/histochemical features that distinguish non-malignant and malignant tissue. However, not all researchers have access to tissue. Lipschutz et al. (25) described a neonatal rat ventral prostate model grown in serum-free medium floating on filters. These rat prostate cultures were shown to grow and differentiate in a manner similar to that of the developing prostate in vitro. This model has consequently been used to study prostate development but may also be useful for cancer research.

Likewise, mammary tissue can also be obtained from non-malignant and malignant human surgical specimens to represent a continuum of mammary cancer progression. However, organ culture is not without problems. There can be difficulty in obtaining specimens and issues with poor tissue viability in culture. Considerable emphasis has been placed on developing non-human 3D models for studying breast development and cancer. The simplest 3D culture is monotypic 3D culture where single cell types typically isolated from mice are grown in a complex substrate such as Matrigel (BD Biosciences, Franklin Lakes, NJ). Mammary epithelial cells grown in this environment adopt a spherical shape, polarize and are capable of mammary specific function (26). These monotypic cultures ignore the contributions of other cell types to cancer progression. Development of organotypic co-cultures more closely approximate the heterogeneous cell populations present in tissue and allow for studying the contributions of these cell populations. For extensive review of these 3D culture systems, see reviews (26–28).

Complex culture systems approximate the microenvironment of tissue and allow for a more physiologically relevant model to study carcinogenesis and the stage-dependent effects of dietary compounds on this process. These models, while technically challenging to develop, may be better suited to elucidate complex, heterotypic interactions between cells during carcinogenesis. Arguably, cell culture models are better suited to studying the cellular processes and signaling pathways involved in oncogenic transformation of epithelial cells and how dietary compounds may inhibit these processes at the cellular level. The following discussion will review relevant cell lines that may also serve as models for cancer chemoprevention research.

Normal primary human cells and cell lines

Human primary cells, isolated from surgical specimens, provide a highly relevant model system to study early events in carcinogenesis. However, acquiring these surgical specimens is difficult as it necessitates physician/hospital collaboration with relatively constant tissue availability, human/tissue use agreement forms, and biosafety Level II training and facilities for handling human tissue. In addition, tissue availability can be unpredictable, and specimen isolated primary cells from normal tissue and stages of tumor tissue require technical expertise and delicate culture conditions, and only survive for a few replications. Often, experimental results using primary cells can vary dramatically by individual donor because the cells of various stages are isolated from different individuals and are probably not to be syngeneic (identical/very similar genetic makeup). This lack of similar genetics between different samples is a critical limitation of isolated primary human cells. However, these primary cell lines can provide powerful insights into cancer biology relevant to human cancer. As an illustrative example, this type of primary cell culture was recently used to compare the autofluorescence of normal, hyperplastic and adenomatous colon mucosa in the hopes of identifying a biomarker useful during endoscopy (29).

One way to overcome surgical specimen limitations is to purchase commercially available immortalized normal human epithelial cells (examples: Incell Corp. or Cambrex Clonetics). Ideally, these normal cell lines could then be altered experimentally to induce genetic mutations or be transfected to overexpress genes of interest in cancer progression. Some companies will allow this type of genetic modification to their proprietary cell lines and others will not. However, purchasing these cell lines requires licensing/material and publication agreements that must be approved by institution-specific legal offices. Additionally, these cell lines are resource-intensive, licensing fees are expensive, the cells are costly and the cell lines typically require proprietary media and supplements for maintenance. In addition, the cell lines typically have a finite lifespan. For example Clonetics will guarantee the cells for 10–15 population doublings, which equates to about 2–3 passages. Different lots of cells can have different results owing to donor differences; so to ensure uniformity across assays Clonetics recommends the need to specify the same lot as long as it is available (information available at Cambrex web site www.cambrex.com).

The following are examples of how these commercial cell lines are being used in cancer prevention research. Clonetics cells have been used to understand differing responsiveness of ovarian epithelial cells to growth factors or steroids that may relate to their susceptibility to malignant transformation (30). NCM460 [non-tumorigenic colon epithelial cells (CECs) available from Incell Corporation, San Antonio, TX] and Caco-2 (colon adenocarcinoma cells available from American Type Culture Collection Manassas, VA) cells were used to compare the growth response of normal and colon cancer cells to doses of anthocyanin-rich extracts isolated from grapes, bilberry and chokeberry (31). The authors observed greater growth inhibition of colon cancer cells, as compared to non-tumorigenic colon cells by these extracts. This example highlights the utility of comparing the effects of dietary compounds on different stages of cancer cells. However, this model also suffers from the limitation that these two cell lines were not syngeneic (i.e. did not share similar genetic backgrounds or were not isolated from that same individual).

Modeling epithelial cancer progression in vitro: breast models

Another approach to modeling stages of cancer progression is to use immortalized cell lines. Many cancer cells isolated from tumors are immortal in culture and simple to maintain and are not limited by passages. Other ‘more normal’ cell lines are isolated from tissue originally but then can be made conditionally immortal by insertion of genes regulating cell growth like SV40 large T antigen. Cells may also be passaged in culture
until cells experience spontaneous mutations that can result in cellular immortality. These non-tumorigenic cell lines can then be transfected with genes of interest. These types of cell lines are typically inexpensive or possibly gifted from the investigator who developed the cell line, easy to maintain in culture and do not require licensing agreements/fees.

Three immortalized breast epithelial cell lines have been used to compare the stage-specific effects of a Chinese herb on the growth and expression of three breast cancer cell lines: MCF-10A, MCF-7 and MDA-MB-231 (32). MCF-10A cells are spontaneously immortalized non-transformed normal mammary epithelial cells derived from a 36-year-old patient with fibrocystic changes (33). MCF-7 cells are human mammary adenocarcinoma cells that are non-metastatic and weakly motile. Finally, the MDA-MB-231 cells represent highly malignant, poorly differentiated human breast adenocarcinoma cells (34). These are examples of several isolated immortal breast epithelial cell lines available and are used comparatively to study cell biology, including chemotaxis related to normal and cancer cell growth control.

Burdall et al. (35) discuss the pros and cons of established cell lines versus primary cell isolation in a recent review, with emphasis on metastatic breast cell line contamination by HeLa cells. They highlight the essentiality of understanding the limitations of the model selected and taking known limitations into consideration when designing experiments and interpreting results (35). More recently established novel breast cell carcinoma lines (BCC), especially those established from primary breast tumors, are worth consideration, mindful that primary culture offers a more relevant clinical model of this disease that is likely to provide more meaningful data (35). In a recent review of BCC, Lacroix and Leclercq (36) concluded that BCC lines are likely to reflect, to a large extent, the features of cancer cells in vivo. They emphasized the importance of estrogen receptor-α and Her-2/neu as characteristics of breast cell lines with biological relevance. Finally, they suggest development of a larger set of cell lines since the origin of some of the widely used lines remains ambiguous. In another review, the authors state that breast cell culture models have provided valuable leads for molecular pathogenesis of cancer progression and suggest validation of models as a high-throughput mechanistic screen for preclinical efficacy of natural phytochemicals (37).

**Modeling epithelial cancer progression in vitro: prostate models**

Prostate cancer prevention researchers have access to similar cell lines, most of which have been developed in the last five years. There are more than 200 prostate cancer cell lines established by investigators from primary tissue sources and clonal derivatives of previously established lines. Some of these cell lines were derived by the insertion of transgenes, including human telomerase reverse transcriptase, SV40 T antigen and human papillomavirus genes and are extensively discussed in a two-part review article (38,39). These authors reinforce the point that new established cell lines provide prostate cancer researchers with a critical research resource. Unique to prostate cancer research, the authors also created an online database of these prostate cancer cell lines freely accessible via the World Wide Web at http://www.CaPCellLines.com. The web-based interface allows researchers to collect information regarding cell lines, add new cell lines and update or add new information regarding established cell lines (38,39).

Recently, Sinisi et al. (40) derived a non-transformed differentiated epithelial prostate cell line (epithelial non-transformed prostate cells; ENP) from normal prostate tissue by passing the cells 70 times until a spontaneously immortalized population was established. The authors compared the effect of all-trans-retinoic acid (ATRA) on ENP cells as compared with primary normal and primary malignant prostate epithelial cells isolated from tissue (41). These types of cell lines greatly expand the arsenal of in vitro models available for stage-specific prostate cancer chemoprevention research.

An exemplary application of ATRA to cultured cells and a syngeneic murine model of prostate cancer susceptibility highlights the difficulty in translating in vitro biological activities to decreased murine tumor reduction in vivo. Using the autochthonous spontaneous transgenic adenocarcinoma of the mouse prostate (TRAMP) model system, ATRA decreased total viable cells with a concomitant decrease in cells in S phase in TRAMP-derived C2N prostate tumor cells (42). When TRAMP mice were treated in vivo with ATRA for either 6 or 8 weeks at low, medium or high dose, mice on average presented with lower grade and more differentiated tumors. However, ATRA therapy conferred no significant protection on incidence of tumors or frequency of metastasis at any dose. These examples illustrate the utility of employing mouse models, and cell lines derived from these mice, to provide rich resources for conducting rigorous hypothesis-driven experiments that can yield important insights into stage-dependent effects of dietary compounds on cancer progression.

**Modeling epithelial cancer progression in vitro: intestinal models**

Unfortunately, normal and premalignant primary intestinal epithelial cells that could be used to investigate dietary chemopreventive cell biological effects are difficult to obtain and culture. Cell culture studies have mostly used carcinoma cell lines from various species or rat small intestinal epithelial cells. Some normal immortalized cell lines have been established but are not widely used (NCM460; Incell Corp) and premalignant adenoma cell lines are extremely rare. Three examples of intestinal immortal epithelial cell lines have been used to compare the stage-specific antiproliferative potency of flavonoids: IEC-6, HT-29 and Caco-2 cell lines (43). IEC-6 cells are normal rat small intestine epithelial cells capable of at least 10 population doublings. HT-29 human colonic adenocarcinoma cells are tumorigenic and were isolated from a 44-year-old female. Finally, Caco-2 cells are also human colonic adenocarcinoma that are tumorigenic but were isolated from a 72-year-old male. In this case, the model cell lines being compared are from different species and clearly represent a limitation. To overcome this limitation, researchers are taking the non-tumorigenic intestinal epithelial cell lines like IEC-6 and RIE-1 (rat small intestine normal fetal cell line) and stably transfecting them with genes that impart characteristics more like premalignant and malignant cells. These techniques allow for creating syngeneic cell lines that represent stage-specific cell models of cancer. However, constitutive overexpression of genes does not always represent physiological relevant models of premalignant cells. It is more ideal to isolate cells that possess physiologic gene expression levels.
Conditionally immortal colon epithelial cell lines: an excellent model of early events in colon cancer to study cancer chemoprevention?

We propose that a specific set of non-tumorigenic, conditionally immortal cell lines derived from C57/BL6 mice, YAMC [young adult mouse colon cells (Apc\(^{+/+}\))] cells and IMCE [Immorto-min colonic epithelium cells (Apc\(^{Min/+}\))] cells, developed by Dr Robert Whitehead (Vanderbilt University, Nashville, TN), have yielded important findings on early events in colorectal neoplasia. We believe the cell lines can serve as a model to examine the effect of dietary compounds for prevention of colon cancer on an early preneoplastic stage with a relevant mutation in the adenomatous polyposis coli (APC) tumor suppressor gene. A mutation in Apc is considered the gatekeeper mutation in colon cancer. An inherited mutation in Apc results in a syndrome called familial adenomatous polyposis (FAP) and is mutated in up to 80% of all sporadic colon tumors.

In this section, we will review the development, methodology, utility and limitations of these cell lines for colon cancer prevention research and discuss how these two cell lines can serve as an excellent model for diet and cancer prevention research.

Cell lines. This model system consists of two murine CEC lines, one that mimics normal CECs, YAMC (Apc\(^{+/+}\)), and a cell line with a mutation in the Apc tumor suppressor gene, IMCE (Apc\(^{Min/+}\)), that mimics preneoplastic CECs. This mutation in the Apc gene is very relevant to colon cancer research as it is considered one of the earliest events in the initiation and progression of CRC (44,45).

Derivation of cell lines. The YAMC (Apc\(^{+/+}\)) cell line was developed from the transgenic mouse (Immortal mouse) bearing a temperature-sensitive mutation of the simian virus 40 large tumor antigen gene (tsA58), which was placed under the control of the gamma-interferon-inducible H-2Kb promoter (H-2Kb-tsA58) (46). Colonic crypts were isolated from the Immortal mouse and YAMC (Apc\(^{+/+}\)) cells were collected as described (47). The IMCE (Apc\(^{Min/+}\)) colonic epithelial cell line was derived from an F1 hybrid between the SV40 large T antigen transgenic mouse (Immortal mouse) and the Apc\(^{Min/+}\) mouse (48). Min mice develop intestinal adenomas similar to FAP patients (49). Genetic examination demonstrated that Min mice carry a mutation in the murine homolog of the human APC gene (Apc) (50).

Important properties of the cells. YAMC (Apc\(^{+/+}\)) and IMCE (Apc\(^{Min/+}\)) cell lines display epithelial morphology: they express keratin-18 (an epithelial cell-specific protein), growth in culture is contact-inhibited, and they die over 7 days (46–49). In addition, these cell lines are non-tumorigenic in nude mice, do not grow in soft agar and survive in culture only on ECM proteins such as collagen I (48). When the cell lines are transfected with K-ras, IMCE (Apc\(^{Min/+}\)) cells are able to grow in soft agar and form tumors in nude mice within 3 weeks and YAMC (Apc\(^{+/+}\)) cells form tumors after 90 days (51). The cells are grown on 75 cm\(^2\) culture flasks coated with 5 μg/cm\(^2\) type I collagen in RPMI 1640 media supplemented with 5% neonatal calf serum, insulin/transferrin/selenium (ITS), 5 IU/ml of murine IFN-γ and 100 000 IU/ml penicillin and 100 mg/l streptomycin with 5% CO\(_2\). As described above, both YAMC (Apc\(^{+/+}\)) and IMCE (Apc\(^{Min/+}\)) cells express the heat-labile SV40 large T antigen under the control of an IFN-γ-inducible promoter. At 33°C with IFN-γ present in the media, the temperature-sensitive SV40 large T antigen is active, binds to and inactivates p53 and drives cell proliferation. At 39°C the temperature-sensitive mutation yields an inactive protein, and the cells behave as non-proliferating, differentiated colonic epithelial cells. Under these non-permissive conditions, the cell lines behave like normal cells in that they are contact-inhibited and undergo apoptosis if they achieve maximal confluence. Therefore, conditions are optimized for cells to proliferate slowly for 24 h at 39°C and then undergo cell death over 5–8 days, similar to the life cycle of a normal CEC.

Limitations. These cell lines do not perfectly mimic the characteristics of normal CECs. The cell lines are conditionally immortal because they possess the SV40 large T antigen and as a result the morphology is that of immature epithelial cells. While they do not form a brush border at the apical surface and only a few microvilli are observed, they do make brush border enzymes detectable in culture (46). Further, the IMCE (Apc\(^{Min/+}\)) cell line shares the same limitations as those of the Min mouse. Notably, tumors occur predominantly in the small intestine of the Apc\(^{Min/+}\) mouse, and not the colon, and seldom develop adenocarcinomas (48). Consequently, the aberrant crypt foci-to-carcinoma progression is not established in this model (52). Different from human adenocarcinomas, the K-ras mutations were not detected in Apc\(^{Min/+}\) mouse polyps, and p53 inactivation, frequent in human cancers, does not increase tumors in Apc\(^{Min/+}\) mice (52–54).

Despite these limitations, the cell lines overcome significant difficulties associated with culturing normal epithelial cells. Intestinal cells are very difficult to culture and are therefore difficult to immortalize in culture. Typically, colonic crypt cultures die within 24 h (46). The Immortal mouse-derived cell lines allow for conditional long-term passage of these cell lines and subsequent use in experiments.

A number of investigations have established these cell lines (IMCE and YAMC) as relevant models for use in studies on early events in CRC (see Table II for summary). These in vitro studies have shown that phenomena observable in monoculture
have relevance for unraveling mechanisms related to human CRC. They reproduce many phenotypes observable in vivo and as such are models for early events in colon cancer.

Recent findings. YAMC (Apc<sup>+/+</sup>) cells have previously been used to study both normal and cancer-related CEC phenotypes. YAMC (Apc<sup>+/+</sup>) cells are clearly a model of normal CECs as investigators used this cell line to understand the: (i) regulation of CEC proliferation/apoptosis (55–57), (ii) role of p53 (58), (iii) role of growth factors on CEC migration (59,60), (iv) regulation of β-catenin and iNos/Cox-2 in CECs (61–63), (v) effect of bacteria on CECs (64–66) and (vi) biological effects of compounds on CECs (67–70). In addition, YAMC (Apc<sup>+/+</sup>) cells are used to model inflammatory bowel disease by treating the cells with TNF-α and understand the effects on CECs and tumorigenesis (71–76). Still other investigators have used YAMC (Apc<sup>+/+</sup>) cells to model normal and transformed cells concurrently by testing the effects of compounds on these cells under either permissive 33°C (immortal) or growth-arrested 39°C (normal) conditions (77,78).

In contrast, the IMCE (Apc<sub>Min</sub><sup>+/+</sup>) cells have primarily been used to understand the effect of an APC mutation on a variety of biochemical processes related to colon cancer progression (79,80). One way this has primarily been accomplished is by comparing effects of regulated expression of other genes such as K-ras, β-catenin and KLF-5 (51,81–83).

Each of these studies identifies a manipulable carcinogenesis-related phenotype that may be modulated by dietary compounds. Recently, researchers have begun to use these cell lines as a model to study the effects of purported dietary chemopreventive agents. The YAMC cell line was used to study the possible colon epithelial chemopreventive effects of n-3 polyunsaturated fatty acids (84,85).

Applications. Relatively few researchers have used these cells together in experiments to compare and contrast the effect of a purported dietary chemopreventive agent between heterotypic models of normal versus preneoplastic or neoplastic cells. We present several illustrative examples in Table III of how researchers have employed comparative cell models isolated from breast, colon and oral epithelial cells for the study of phenotypes relevant to the prevention of carcinogenesis by dietary compounds.

We have currently published three examples of how these cell lines can be used in this type of model system. We show that the proposed dietary chemopreventive agents curcumin and specific flavonoids can induce cell migration in an Apc genotype-independent manner (86,87). The data suggest a potential mechanism by which these compounds may exert their chemopreventive effects in the colon by inducing migration in cells heterozygous for Apc to overcome defective cell migration, a phenotype associated with cell differentiation and apoptosis. These examples provide a strong evidentiary basis for continuing efforts to develop in vitro models to assess the preclinical efficacy of dietary compounds for the prevention of biological events associated with the progression of carcinogenesis.

How might these in vitro models be employed to draw inferences about the preventive efficacy of dietary compounds? In short, to answer questions appropriate to the hypothesis and stage of carcinogenesis being addressed in the study. For example, investigators may hypothesize that the application of dietary compound X will prevent mitogenic signaling from growth factor Y that is associated with cell proliferation. This hypothesis is best tested by comparing the response of normal cells (or a model of normal cells) to preneoplastic cells isolated from the organ relevant to this dietary compound. The efficacy of dietary compound X in blocking this signal in both normal and preneoplastic cells may reflect the ability to block the normal to preneoplastic transformation. If dietary compound X blocks growth factor Y

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Exposure (dietary compound)</th>
<th>Observed effects</th>
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<tbody>
<tr>
<td>MCF10A versus MCF10CA1a (Non-tumorigenic versus Tumorigenic breast epithelial cells)</td>
<td>Indole-3-carbinol (I3C)</td>
<td>I3C-induced apoptosis in MCF10A but not in MCF10CA1a cells (92).</td>
</tr>
<tr>
<td>YAMC (Apc&lt;sup&gt;+&lt;/sup&gt;/Apc&lt;sup&gt;+&lt;/sup&gt;) versus IMCE (Apc&lt;sub&gt;Min&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;/Apc&lt;sub&gt;Min&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;) (Non-tumorigenic conditionally immortal murine colonic epithelial cells contrasting in Apc genotype derived from Immorto mouse and Immorto mouse/Min hybrids)</td>
<td>Butyrate (Colonic fermentation product of non-starch polysaccharides)</td>
<td>Butyrate restored normal cell motility to preneoplastic cells (IMCE) (93).</td>
</tr>
<tr>
<td>C57CO versus 1638N COL (Normal human prostate epithelial cells versus malignant primary prostate epithelial cultures)</td>
<td>9-cis-retinoic acid (9cRA)</td>
<td>9cRA caused growth arrest in 1638N COL but not C57CO (94).</td>
</tr>
<tr>
<td>EPN versus NPEC (Normal human prostate epithelial cells versus malignant primary prostate epithelial cultures)</td>
<td>ATRA</td>
<td>RA induced growth arrest and apoptosis in NPEC (malignant) cells (95)</td>
</tr>
<tr>
<td>Normal versus premalignant versus carcinoma cells (Primary cultures of normal oral epithelial cells, newly established cell lines derived from dysplastic leukoplakia and squamous cell carcinoma)</td>
<td>(+)-epigallocatechin-3-gallate (EGCG) and curcumin</td>
<td>EGCG and curcumin, alone and in combination, decreased cell cycle progression more effectively in carcinoma cells than normal or dysplastic cells (96).</td>
</tr>
</tbody>
</table>
signaling in preneoplastic cells then the only inference that can be drawn is that this compound prevents preneoplastic cell progression but not the transformation from normal to preneoplastic cells. Another example of a hypothesis that can be tested in vitro is that dietary compound X decreases metastatic potential of tumor cells by altering the composition of stroma in the microenvironment of the tumor. This hypothesis requires a complex organ culture model in which the effect of the dietary compound on the production of ECM by heterogeneous cell types can be assessed. The decreased metastatic potential of tumor cells in this model can be associated with both concentration of dietary compound X and the associated changes in stromal ECM composition. Selection of a hypothesis-appropriate model will facilitate the strength of preventive inferences drawn from in vitro studies and shed light on stage-specific biological mechanisms of putative dietary chemopreventive compounds.

Investigators acknowledge a need for the development of in vitro cell culture and organ systems that model the continuum of normal to tumor cell transition. Current in vitro model systems are limited by biological as well as logistical obstacles. Undoubtedly, these more elaborate models will lead to elucidation of biological mechanisms for purported dietary chemopreventive agents identified in human observational studies and begin to explain the perplexing data from randomized controlled clinical trials.

Is the past prescient? How can in vitro models of neoplastic transformation inform human chemoprevention

A final illustrative example is provided as a reminder of the potential utility of in vitro stage-specific models to help inform the design of dietary cancer chemoprevention studies. Case–control studies of phytoestrogen intake and lung cancer risk (88–90) consistently show a protective effect. The authors recommend confirmation of these findings by conducting large-scale longitudinal studies. Yet, on the basis of these data, we cannot assess when or how during lung cancer progression these purported chemopreventive phytoestrogens affect risk. Because of the limited nature of the case–control evidence associating isoflavone intake and decreased lung cancer risk, we suggest that caution be applied before large, expensive prospective studies are engaged. In epidemiology, establishing near-certain causal relationships implies highly consistent statistically significant results across many different studies, large relative risk estimates, extensive understanding of biological mechanisms and dose–response relationships, positive prevention trial results, a clear temporal relationship between cause and effect and other conditions spelled out in terms of the widely used causal criteria (91). It is hoped that, in the interests of the rigorous application of the principles of causal inference, additional causal criteria besides biological plausibility be met prior to this investment in randomized, controlled clinical trial.

The large-scale longitudinal studies suggested above would presumably involve supplementation of individuals at high risk for lung cancer (e.g. smokers) with soy isoflavones. However, on the basis of recent chemoprevention trials discussed above, there is the distinct possibility that isoflavone supplementation in smokers may result in increased risk of developing lung cancer. We hypothesize that investigators may, in fact, discover that soy isoflavones promote lung tumor cell progression and growth in a similar manner shown for isoflavone’s potential promotional effect on breast cancer.

It is critical to develop and utilize valid reductionist model systems for describing the differential effects of dietary compounds on disease progression before undertaking large public investments (and potential risks) in chemoprevention trials. Model in vitro systems are rapidly being established by researchers that will provide tools for cancer prevention researchers to employ and allow us to gain a better understanding of the stage-specific effects of purported dietary compounds on cancer chemoprevention. These model systems, while more often exploited by basic cancer biologists, have been underserved in the nutrition and cancer biology chemoprevention field. The use of syngeneic cell culture model systems may yield important insights into the biological mechanism and specific stage at which a dietary compound may have its most beneficial effect on cancer prevention and potentially inform animal model and eventually human studies of diet and cancer chemoprevention.

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References


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