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Suppression of Chronically Induced Breast Carcinogenesis and Role of Mesenchymal Stem-like Cells

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I am submitting herewith a dissertation written by Kusum Rathore entitled "Suppression of Chronically Induced Breast Carcinogenesis and Role of Mesenchymal Stem-like Cells." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

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Suppression of Chronically Induced Breast Carcinogenesis and Role of Mesenchymal Stem-like Cells

A DissertationPresented for theDoctor of PhilosophyDegree

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Kusum Rathore

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DEDICATION

This dissertation is dedicated to my Grandparents, Late Shri Lal Singh Rathore,

Late Sri Ram Singh Bhati and Late Shrimati Kamla Kumari

with love.

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--Kusum Rathore

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Abstract

Sporadic breast cancers are mainly attributable to long-term exposure to environmental factors, via a multi-year, multi-step, and multi-path process of tumorigenesis involving cumulative genetic and epigenetic alterations in the chronic carcinogenesis of breast cells from a non-cancerous stage to precancerous and cancerous stages. Epidemiologic and experimental studies have suggested that various dietary compounds like green tea and grape seed may be used as preventive agents for breast cancer control. In this research, I have developed a cellular model that mimics breast cell carcinogenesis chronically induced by cumulative exposures to low doses of environmental carcinogens. I used the chronic carcinogenesis model as a target system to investigate the activity of dietary compounds at non-cytotoxic levels in intervention of cellular carcinogenesis induced by cumulative exposures to pico-molar 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a]P). I used various cancer-associated properties like, reduced dependence on growth factors, anchorage-independent growth, increased cell mobility, and acinar-conformational disruption as measurable endpoints of carcinogenesis.

The first part (Part-I) of this dissertation focuses on the understanding the breast cancer progression, importance of environmental carcinogens, role of diet in cancer prevention and importance of epithelial to mesenchymal transition and stem-like cells in chronic carcinogenesis. The next three parts (Part II-IV) focus on understanding the role and mechanisms of dietary compounds in prevention of carcinogenesis and stem-like cell properties. Results in part II revealed the green tea extract at bio-achievable concentration can suppress carcinogen-induced cancerous properties. In Part-III, I compared the four major catechins in green tea extract in suppressing chronic carcinogenesis and the results revealed that epicatechin gallate to be most

effective. I also identified that short-term exposure to NNK and B[a]P resulted in elevation of reactive oxygen species, ERK pathway activation and induction of cell proliferation and DNA damage, which can be blocked by green tea catechins. Results in Part-IV describe the roles of properties and markers associated with stem-like cells and the epithelial to mesenchymal transition induced by chronic carcinogenesis and their suppression by green tea catechins and grape seed proanthocyanidin extract. The last section (Part-V) summarizes the findings with their importance and discusses future directions.

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ABBREVIATIONS

ATCC	American Type Culture Collection
ANOVA	A one-way analysis of variance
B[a]P	Benzo[a]pyrene
BrdU	5-bromo-2'-deoxyuridine
CDK	Cyclin-dependent kinase
CM-H2DCF-DA	Chloromethyl-dichlorodihydrofluorescin-diacetate
CM medium	Complete MCF10A medium
EC	Epicatechin
ECG	Epicatechin-3-gallate
EMT	Epithelial to mesenchymal transition
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallate
ELISA	Enzyme-linked immunosorbent assay
EpCAM	Epithelial cell adhesion molecule
ERK	Extracellular signal regulatory kinase
FITC	Fluorescein isothiocyanate
GTCs	Green tea catechins
H_2O_2	Hydrogen peroxide
IC_{50}	50% inhibitory concentration
LM medium	Low-mitogen medium

MMP-9	Matrix metallopeptidase 9
MTT	Methyl Thiazolyl Tetrazolium xi
NAC	N-acetyl-L-cysteine
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
PBS	phosphate buffer saline
PE	Phycoerythrin
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase

PART-I

Background and Overview

Breast Cancer

According to American Cancer Institute, cancer is a group of diseases that cause cells in the body to change and grow out of control. Breast cancer begins in breast tissue, which is made up of glands for milk production, called lobules, and the ducts that connect lobules to the nipple. The remainder of the breast is made up of fatty, connective, and lymphatic tissue [1]. Breast cancer (malignant breast neoplasm) is cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. Cancers originating from ducts are known as ductal carcinomas; those originating from lobules are known as lobular carcinomas. Breast cancer is the most common type of cancer among women in northern America and northern Europe. National Institute of Health estimates 230,480 (female); 2,140 (male) new cases and 39,520 (female); 450 (male) deaths from breast cancer in the United States in 2011 [2]. Worldwide, breast cancer comprises 22.9% of all cancers in women [3]. Rates of breast cancer around the world vary a great deal, with industrialized countries generally having higher rates than non-industrialized countries [4] (**Figure 1.1**).

Stages of Breast Cancer

Staging is the process of determining how much cancer there is in the body and where it is located. The underlying purpose of staging is to describe the severity and extent of an individual's cancer, and to bring together cancers that have similar prognosis and treatment [5]. Staging of breast cancer is one aspect of breast cancer classification that assists analyzing severity and in making appropriate treatment choices.

As classified by American Cancer Institute [6] the main stages of breast cancer are (**Figure 1.2**):

Stage 0 (carcinoma *in situ*)

This is *in situ* disease or Paget's disease of the nipple. Stage 0 is a pre-cancerous condition, and is called carcinoma *in situ*. There are 2 types of breast carcinoma *in situ*:

Ductal carcinoma *in situ* (DCIS) is a noninvasive condition in which abnormal cells are found in the lining of a breast duct. The abnormal cells have not spread outside the duct to other tissues in the breast. In some cases, DCIS may become invasive cancer and spread to other tissues, although it is not known at this time how to predict which lesions will become invasive.

Lobular carcinoma *in situ* (LCIS) is a condition in which abnormal cells are found in the lobules of the breast. This condition seldom becomes invasive cancer; however, having lobular carcinoma *in situ* in one breast increases the risk of developing breast cancer in either breast.

Stage 1

During stage I, cancer has formed. Stage I is divided into stages IA and IB.

In stage IA: The tumor is 2 centimeters or smaller and has not spread outside the breast.

In stage IB: No tumor is found in the breast, but small clusters of cancer cells (larger than 0.2 millimeters but not larger than 2 millimeters) are found in the lymph nodes.

Stage II

Stage II is divided into stages IIA and IIB.

In stage IIA: No tumor is found in the breast, but cancer is found in the axillary lymph nodes (lymph nodes under the arm); or the tumor is 2 centimeters or smaller and has spread to the axillary lymph nodes; or the tumor is larger than 2 centimeters but not larger than 5 centimeters and has not spread to the axillary lymph nodes.

In stage IIB: The tumor is either larger than 2 centimeters but not larger than 5 centimeters and has spread to the axillary lymph nodes; or larger than 5 centimeters but has not spread to the axillary lymph nodes.

Stage III

Stage IIIA: No tumor is found in the breast. Cancer is found in axillary lymph nodes that are attached to each other or to other structures, or cancer may be found in lymph nodes near the breastbone; or the tumor is 2 centimeters or smaller.

Stage IIIB: The tumor may be any size and cancer has spread to the chest wall and/or the skin of the breast; and may have spread to axillary lymph nodes that may be attached to each other or to other structures or cancer may have spread to lymph nodes near the breastbone.

Cancer that has spread to the skin of the breast is inflammatory breast cancer.

Stage IV

Stage IV describes invasive breast cancer that has spread beyond the breast and nearby lymph nodes to other organs of the body, such as the lungs, distant lymph nodes, skin, bones, liver, or brain. This is the metastatic stage.

Risk factors for Breast cancer

A risk factor is anything that increases a person's chance of getting a disease. Different cancers have different risk factors. As stated by Breast Cancer Organization [7], there are some known risk factors for breast cancer (**Figure 1.3**). Just being a woman is the biggest risk factor for developing breast cancer. There are about 190,000 new cases of invasive breast cancer and

60,000 cases of non-invasive breast cancer this year in American women. As with many other diseases, risk of breast cancer goes up with age. About two out of three invasive breast cancers are found in women 55 or older.

Women with close relatives who've been diagnosed with breast cancer have a higher risk of developing the disease having one first-degree female relative (sister, mother, daughter) diagnosed with breast cancer, doubled the risk. About 5% to 10% of breast cancers are thought to be hereditary, caused by abnormal genes passed from parent to child. Radiation exposure to the chest to treat another cancer, such as Hodgkin's disease or non-Hodgkin's lymphoma, also increases the average risk of breast cancer. Being diagnosed with certain benign breast conditions also increases risk of breast cancer. Caucasian women are slightly more likely to develop breast cancer than African-American, Hispanic, and Asian women. But African-American women are more likely to develop more aggressive, more advanced-stage breast cancer that is diagnosed at a young age.

Overweight and obese women have a higher risk of being diagnosed with breast cancer compared to women who maintain a healthy weight, especially after menopause. Being overweight also can increase the risk of the breast cancer recurrence in women who have had the disease. Other risk factors include using HRT (Hormone Replacement Therapy), drinking alcohol, lack of exercise and smoking.

Studies show that only 3-10% breast cancer is genetic, more that 85% is non-genetic breast cancer caused by environmental factors [8], so it is important to study and understand the role of such environmental factors in breast cancer development

Environmental Carcinogens

Environmental carcinogens are defined as any of the natural or synthetic substances that can cause cancer. Humans are subjected to a range of chemical exposures from the environment. Chemicals in air, water, soil and food, occupational exposures and lifestyle factors, all contribute to a complex exposure situation in our daily life [9]. More that 90% are non-hereditary or 'sporadic' cancers [8]. Considering the importance of chronic exposure of human tissues environmental carcinogens in causing most sporadic human cancers, it is imperative to study, understand and prevent the factors associated with causing sporadic cancers.

According to the American Cancer Society [10] some of the non-hereditary potential cancer-causers (**Figure 1.4**) can be classified as following:

- Related to lifestyle (smoking, alcohol, diet, being sedentary)
- Naturally occurring substances (ultraviolet rays, infectious agents, radon)
- Chemicals in the home and workplace
- Medical treatments (hormone replacement, immune-suppressing treatments)
- Others

Related to lifestyle

Most of the lifestyle-related factors are not by themselves cancer causing agents, but are risk factors associated with the genesis of cancer, but occupational exposures, behavior-related habits and addiction can lead to exposure to higher dose of carcinogens [11]. It is well agreed that smoking and to a lesser extent alcohol consumption, diet imbalance, obesity and lack of physical exercise can contribute to cancer in high-income countries [11].

Tobacco smoking

Smoking is indeed a serious concern, and a major risk factor contributing to human carcinogenesis in multiple organs [12]. Tobacco smoke contains thousands of compounds including many mutagens such as polycyclic aromatic hydrocarbons and nitrosamines and as well as other promoters. Therefore this mixture constitutes the equivalent of what is defined as a complete carcinogen [13]. As shown by multiple studies, smoking is a risk factor for several types of cancers, mainly lung cancer and cancers of the upper aero digestive tract, and also, to a certain extent, for esophagus, stomach, pancreas, liver, bladder, kidney, mammary and cervical cancers, as well as myeloid leukemia [12,14]. Because many of these cancers are associated with a poor prognosis, smoking remains a major cause of cancer mortality.

Alcohol consumption

Ethyl alcohol is not per se a mutagenic agent for DNA, but rather acts mainly as a co-carcinogen. On the basis of epidemiological data, alcohol has been classified as a human carcinogen [15]. Studies have shown that alcohol can potentiate the carcinogenic effects associated with smoking or other related risk factors [16], but overall its action is not clear.

Diet

Several studies have shown that in developed countries, food intake imbalance which is rich in calories and animal fat and low in fiber, is a factor that leads to the occurrence of some cancers (colon, prostate, endometrium and breast) and conversely a high intake of fruits and vegetables with high antioxidant contents have a protective anticancer effect [17,18]. A very important dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is one of the most mass-abundant heterocyclic amines, which are a group of mutagenic compounds

particularly found in high-temperature cooked meats, such as grilled/barbecued meats [19-21]. Epidemiological studies indicate that consumption of well-done meats containing PhIP may play an important role in the risk of breast and other cancers [22].

Overweight, obesity and sedentary lifestyle

Overweight, obesity and sedentary lifestyle have been incriminated as risk factors for cancer [23, 24]. Studies in the USA have suggested that obesity associated with some cancers can worsen cancer mortality [25]. An According to American Institute for Cancer Research study where they looked at seven cancers with known links to obesity and calculated actual case counts that were likely to have been caused by obesity. The results showed that 49% of endometrial cancers are associated to excess body fat. That number is followed by 35% of esophageal cancer cases; 28% of pancreatic cancer cases; 24% of kidney cancer cases; 21% of gallbladder cancer cases; 17% of breast cancer cases; and 9% of colorectal cancer cases [26].

Naturally occurring substances

Ultraviolet rays

Though most forms of radiation have not been linked to cancer, there is evidence that certain types of radiation, such as ionizing and ultraviolet, can damage DNA and cause cancer. UV irradiation is one of the most relevant risk factors for the development of skin cancer [27-29]. The UV-induced tumors show a rapidly rising incidence not only in the United States but also in Europe and in Australia [30].

Infectious agents

Research indicates that 15 to 20 % of worldwide cancer cases are related to infections caused by viruses, bacteria and parasites [31]. Some infections can cause inflammation, which may suppress a person's immune system or directly affect a person's DNA. Bacteria such as *H. pylori* implicated in a greater risk for stomach cancer [32] and viruses such as HPV linked to cervical cancer [33] can increase risk for developing cancer.

Radon

Radon, a colorless, odorless, radioactive gas, is among the best studied of environmental carcinogens. It is found in soil and rock, outdoors and indoors, with the highest levels in basements, where radon leaks through cracks or gaps in floors or walls. High levels of radon have been linked to lung cancer [34]. These high levels can occur when radon becomes concentrated in an area and cannot dissipate. Exposure to radon is the second leading cause of lung cancer, after smoking. In the US, radon causes about 21,000 lung cancer deaths every year out of a total of about 160,000 annual lung cancer deaths [35].

Chemicals in the home and workplace

According to National Institute of Environmental Health Sciences, any substances and circumstances that are "known" or are "reasonably anticipated" to cause cancer are considered chemical carcinogens. A large number of chemicals are considered carcinogenic. The list includes benzene, auto exhaust, cigarettes, industrial processes and some consumer goods, which have been linked to various types of cancers. Many other environmental substances are potentially carcinogenic and under study to determine their level of cancer-causing potential as

listed by the Environmental Protection Agency and American Cancer Society [36]. A few important chemical carcinogens are discussed in detail here.

Diesel exhaust: A contributor to air pollution has been classified as an environmental carcinogen [37] and is most associated with an increased risk of lung cancer. It is given off by vehicles, construction and farm equipment with diesel engines [38]. The people with the highest exposure are at the greatest risk of developing cancer [39].

Secondhand smoke: Also known as passive smoke, secondhand smoke contains more than 4,000 chemical compounds, with more than 60 of these compounds known or suspected to cause cancer [40]. Secondhand smoke is most dangerous in enclosed places, such as home, car, workplace and public buildings where smoking indoors is allowed. Secondhand smoke exposure has been shown to be associated with increasing incidences of lung and other types of cancer [41].

Asbestos: found in older buildings, increases the risk of lung, malignant mesothelioma of the pleura and peritoneum, cancer of the larynx, and certain gastrointestinal cancers [42]. The total deaths in US due to asbestos-related lung cancer, mesothelioma and asbestosis from workplace exposure resulted is estimated to be over 200,000 for year 2010 [43]. In addition, several thousands of deaths can be attributed to other asbestos-related diseases, as well as to non-occupational exposures [44].

Medical treatments (hormone replacement, immune-suppressing treatments)

Hormone replacement therapy (HRT) has been used extensively in postmenopausal women as a proven and effective therapy for climacteric symptoms and in osteoporosis

treatment. Its widespread long-term use for indications such as primary prevention of cardiovascular disease or cognitive decline was called into question with the publication of several epidemiological studies [45]. The risk of breast cancer varies with the formulation and preparation of HRT. Opposed estrogens (progesterone–estrogen) in oral form are associated with an increased risk of breast cancer, which increases with use [46]. HRT has also shown to cause increased risk of ovarian [47], colorectal [48], brain [49] and other types of cancer.

Immunosuppressive mediations are essential to prevent graft rejection and to optimize graft survival. However, because these medications suppress the immune system, whose main function is to fight off infection and prevent the development of cancer, transplant recipients are at elevated risk for infection and certain cancers [50, 51]. The exact mechanism by which immunosuppressive medications promote tumor growth is currently being studied. However, several lines of evidence suggest the duration, intensity and type of immunosuppressant may be related to the development of skin cancer [52], lymphoma [53] and some other types of cancer. The risk of cancer with these treatments greatly depend on the dose, therefore talking to the doctors before starting such therapies is the best way to understand the personal risk of cancer.

Others

There are many other environmental factors which have and can cause cancers. Environmental Protection Agency report, based on emissions data from 2002, estimates the local and regional concentrations of 181 different air pollutants from vehicles and industrial contaminants; as well as other sources [54]. Particulate materials that cause cancer include powdered metallic cobalt and nickel, and crystalline silica (quartz, cristobalite, and tridymite) [55]. According to International Agency for Research on Cancer [56] other carcinogens included,

antiperspirants, talcum powder, hair dyes, cosmetics as well as food products have shown to be potential carcinogens [57], but the exposure to most of the chemicals is needed to be at occupational level to show significant affect [58]. The use of chemicals at home and work place should be monitored and used only as needed for reducing the risk of excessive exposure to them.

This classification of various environmental carcinogens is only indicative, as many other factors may play role in cancer development.

In this study, the impact of two environmental carcinogens was of interest, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a]P) as affecting breast cancer. These carcinogens are discussed in detail in following sections.

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

Carcinogenic transformation of breast cells from non-cancerous to precancerous and cancerous stages is a multiyear, multistep, and multipath disease process with progressive genetic and epigenetic alterations [59, 60]. Although numerous factors have been postulated to contribute to breast cancer, more than 85% of breast cancers are attributable to environmental factors, such as exposures to chemical carcinogens, dietary habits, etc. [61-68]. Growing evidence indicates exposures to tobacco and environmental carcinogens may increase the risk of sporadic human breast cancer [59, 63–70]; however, the role of tobacco and environmental carcinogens in the development of human breast cancer is still not clear. Possibly, the nature of a long-term, accumulative exposure to tobacco and environmental carcinogens plays an important role in the progressive development of human breast cancers. Thus, it is important to understand the ability of tobacco and environmental carcinogens to chronically transform breast epithelial cells in order to clarify their roles in the development of human breast cancer.

Nicotine-derived nitrosamine ketone (NNK), or 4-(methylnitrosamino)- 1-(3-pyridyl)-1-butanone (**Figure 1.5a**), is a nitrosamine present in tobacco that is a potent pro-carcinogen. The tobacco specific carcinogen NNK, one of the most potent carcinogens in tobacco products, induces a high incidence of pulmonary cancers but no breast cancer in laboratory rodents [71,72]. The formation of DNA-adducts from reactive NNK metabolites causes activating point mutations of genes, such as the Ki-ras gene that is frequently observed in human lung cancer cells [71,72]. Recently, Zimmerman's groups have indicated an interchangeable conversion between NNK and its metabolites, suggesting a novel persistent activity for NNK [73]. NNK also acts as a potent agonist for β-adrenergic receptors and α7-nicotinic acetylcholine receptors

and stimulates arachidonic acid-dependent and Erk-dependent mitogenic pathways associated with these receptors [74-78]. Other studies have shown alterations in the cell cycle machinery including cyclin D1, cdk4, p16INK4a, and Rb, contributing to the acquisition of autonomous growth by NNK-induced tumors [79]. However, the association between smoking and breast cancer is still controversial; some studies have indicated there is no influence of smoking on breast cancer incidence [80], and some studies have shown a correlation between them [81]. Recently, a study was conducted by four major Canadian agencies to address this controversy, and the results indicate that active smoking and second-hand smoke increase breast cancer risk [82]. Another recent cohort study in the United States has also shown a connection between smoking and breast cancer in postmenopausal women [83]. Thus, the role of smoking in increasing the incidence of breast cancer should be taken seriously.

Benzo[a]pyrene (B[a]P)

Benzo[a]pyrene (B[a]P), $C_{20}H_{12}$, is a five-ring polycyclic aromatic hydrocarbon (**Figure 1.5b**) whose metabolites are mutagenic and highly carcinogenic. B[a]P is listed as a Group 1 carcinogen by the IARC [14]. B[a]P is found in coal tar, in automobile exhaust fumes (especially from diesel engines), in all smoke resulting from the combustion of organic material (including cigarette smoke), and in charbroiled food. Cooked meat products, regular consumption of which has been epidemiologically associated with increased levels of colon cancer [84] (although this in itself does not prove carcinogenicity)[85], have been shown to contain up to 4 ng/g of benzo[a]pyrene,[86] and up to 5.5 ng/g in fried chicken[87] and 62.6 ng/g in overcooked

charcoal barbecued beef.[88]. Therefore B[a]P is considered a tobacco, environmental, and dietary chemical carcinogen [89-93].

B[a]P intercalates in DNA, covalently bonding to the nucleophilic guanine nucleobases at the N2 position. X-ray crystallographic and nuclear magnetic resonance structure studies show that this binding distorts the DNA [94], inducing mutations by perturbing the double-helical DNA structure. This disrupts the normal process of copying DNA and induces mutations, which explains the occurrence of cancer after exposure. This mechanism of action is similar to that of aflatoxin which binds to the N7 position of guanine [95].

B[a]P has been identified as a mammary carcinogen in rodents [96-98]. Studies have indicated that B[a]P contributes to the development of human breast cancer because its metabolites form strong DNA-adducts that cause DNA lesions [99-102]. However, PAH-derived DNA-adducts alone are insufficient for inducing human breast cancer [59, 99, 102]. Thus, B[a]P may play an important role in precancerous carcinogenesis of human breast cells. The most widely identified precancerous stage of breast cancer is ductal carcinoma-*in-situ* (DCIS). Although DCIS cells have not developed the ability to invade out of the ducts or lobules and metastasize, those cells can develop into and raise the risk of invasive breast cancers [59, 103, 104]. So it is important to identify the role of carcinogens in early precancerous stages of cancer progression in the hope that it will provide better prognosis of the disease and better chance to prevent its progression.

Therefore, further studies are needed to conclusively identify NNK and B[a]P as potent carcinogens in the development of mammary carcinogenesis, especially during the early stages.

Precancerous Model of Cancer Progression

Growing evidence indicates that exposure to environmental carcinogens may increase the risk of sporadic human breast cancer. To address the question of whether NNK and B[a]P are able to induce cellular transformation of human breast epithelial cells, we have taken the approach of mimicking chronic exposure of cells to tobacco and environmental carcinogens by repeated treatments of the non-cancerous human breast epithelial MCF10A cell line with NNK and B[a]P at picomolar concentrations, which mimics doses commonly detected in smokers [105-107]. We have been testing a cellular model that is able to reveal the potency of an environmental carcinogen to induce chronic carcinogenesis of breast cells to progressively acquire identifiable cancer-related properties, mimicking long-term exposure of breast cells to low doses of an environmental carcinogen. Using this model we repeatedly exposure immortalized, noncancerous, human breast epithelial MCF10A cells to B[a]P and/ or NNK)at picomolar concentrations, which results in induction of cancer-related cellular properties [108-112]. This model aims to mimic the chronic exposure to tobacco and environmental carcinogens that are encountered by human on a day to day basis. This model also highlights the importance and relevance of “pre-cancerous” stage for cancer prevention target, rather than the therapeutic approach for malignant cells. Also it helps to qualify the utilization of MCF10A cell line to qualitatively and quantitatively study cancer progression in early stages of human breast epithelial cells.

Detection of Cancer Progression

Reduced dependence on growth factors

A lack of growth factors causes normal cells to become growth-arrested in the cell cycle and to commit apoptosis; however, aberrantly increased cell survivability acquired to reduce dependence on growth factors can lead cells to tumorigenic transformation [109-112]. Cancer cells do not need stimulation from external signals (in the form of growth factors) to multiply. Normal cells require external growth signals (growth factors) to grow and divide. These signals are transmitted through receptors that pass through the cell membrane. When the growth signals are absent, normal cells stop growing, but cancerous cells can grow and divide without external growth signals.

Anchorage-independent growth

Cell adhesion to extracellular matrixes is important for cell survival in a multicell environment; aberrantly increased cell survivability acquired to promote anchorage-independent growth can promote tumorigenic transformation [113, 114]. The cellular transformation is associated with certain phenotypic changes such as loss of contact inhibition (cells can grow over one another) and anchorage independence (cells form colonies in soft agar). Anchorage independence can be described in the light of primary fibroblasts and many fibroblastic cell lines (e.g. BALB/c3T3, NIH-3T3, etc.) that must attach to a solid surface before they can divide [114]. They fail to grow when suspended in a viscous fluid or gel (e.g. agar or agarose), however when these cell lines are transformed, they are able to grow in a viscous fluid or gel and become anchorage-independent. The ability to grow on "soft agar" is a routine test taken as an indication

that cells with this ability are anchorage independent. Anchorage-independence correlates strongly with tumorigenicity and invasiveness in several cell types, such as small-cell lung carcinoma [115-117].

Acquisition of Acinar-Conformational Disruption

Acinar structures with a hollow lumen and apicobasally polarized cells are important characteristics found in glandular epithelia in vivo; the disruption of an intact glandular structure is a hallmark of epithelial cancer even at precancerous stages such as DCIS [118, 119]. Parental MCF10A cells mainly form regular, round spheroids on Matrigel cultures, and NNK-and B[a]P-exposed cells form both regular and irregular spheroids, whereas cancerous MCF-7 cells only form irregular spheroids.

Wound Healing

Cancerous cells acquire an increased mobility compared with their normal counterpart cells [120]. When wounded or scratched, cell monolayers respond to the disruption of cell-cell contacts and an increased concentration of growth factors at the wound margin by healing the wound through a combination of proliferation and migration [121-123]; these processes reflect the behavior of individual cells as well as the properties of increased motility. Cancer cells can move through tissues in a variety of different ways. In some cases, an epithelial-to-mesenchymal transition enables cancer cells to acquire fibroblast-like migratory properties. However, it is also becoming apparent that some cancer cells move in an amoeboid way similar to leukocytes [120].Thus increased mobility is a hallmark characteristic of cancerous cells.

Migration and Invasion

The ability of a cancer cell to undergo migration and invasion allow it to change position within the tissues. For example, these processes allow neoplastic cells to enter lymphatic and blood vessels for dissemination into the circulation, and then undergo metastatic growth in distant organs [125]. The capability for invasion and metastasis enables cancer cells to escape the primary tumor mass and colonize new terrain in the body where, at least initially, nutrients and space are not limiting. The acquired capability for invasion and metastasis represents the last great frontier for exploratory cancer research [126]

Role of ERK Pathway in Cancer

Mitogen-activated protein kinase (MAP kinase) cascades transmit and amplify signals involved in cell proliferation as well as cell death. These signal transduction pathways serve as an indicator of the intensity of trafficking induced by various growth factors, steroid hormone, and G protein receptor mediated ligands. Three major MAP kinase pathways exist in human tissues, but the one involving ERK-1 and -2 is most relevant to breast cancer [128]. The Ras/Raf/MEK/ERK cascade couples signals from cell surface receptors to transcription factors, which regulate gene expression and cell cycle progression [129].

ERK Pathway: Binding of growth factors to receptor tyrosine kinases stimulates the autophosphorylation of specific tyrosines on the receptors. The phosphorylated receptor then binds to an adaptor protein called GRB2 which, in turn, recruits SOS (son of sevenless) to the plasma membrane. SOS is a guanine nucleotide exchange factor which displaces GDP from Ras, subsequently allowing the binding of GTP. GTP-bound Ras recruits and activates Raf. Raf initiates a cascade of protein phosphorylation by first phophorylating MEK. Phosphorylated MEK in turn phosphorylates ERK. Phosphorylated ERK moves from the cytoplasm into the nucleus where it subsequently phosphorylates a number of transcription factors, including the specific transcription factor called Elk-1. Phosphorylated transcription factors turn on transcription (gene expression) of specific sets of target genes. The activity of Ras is limited by the hydrolysis of GTP back to GDP by GTPase activating proteins (GAP) [129] (**Figure 1.6**).

Sivaraman et al. [130] provided the first demonstration of MAP kinase activation in human breast cancer tissues. They compared primary breast cancers with benign tissues using substrate based MAP kinase enzyme assays as well as western blotting methods. All 11 breast cancers had markedly elevated levels of activated MAP kinase. Salh et al. [131] examined 23

human breast cancers by substrate enzyme assay as well as by immunologic techniques. Over expression of both ERK-1 and -2 were demonstrated with use of specific antibodies. Immunoprecipitation studies with anti-activated ERK antibodies demonstrated up to a 2.5-fold increase in activated MAP kinase in breast cancers. However, elevations were only seen in 50% of tumors. Mueller et al. [132] studied 131 breast cancers and 18 normal tissues adjacent to tumors. They reported enhanced activation of MAP kinase in a subset of tumors. There was a trend for higher MAP kinase activity in primary tumors of node positive patients than in those from node negative patients. Activated MAP kinase demonstrated a trend to be predictive of disease free survival. Another study [133] investigated the mechanisms for up-regulation of MAP kinase in breast tumors. They found elevated activated MAP kinase in 11/20 cancers. These data suggest that if the appropriate receptors are present, upregulation of ligands may be responsible for MAP kinase increases in human breast cancers. This possibility is supported by the in vitro studies of Xing and Imagawa [134] who demonstrated that EGF could increase activated MAP kinase in primary cultures of mouse mammary cells. These studies, taken together, suggest that further correlation of activated MAP kinase with the biologic characteristics of tumors is now warranted [128]. Studies show that high dose exposure to environmental carcinogens lead to ERK pathway activation [135], but whether low dose of environmental carcinogens can induce ERK pathway in breast cancer needs to be evaluated. Further work will be needed to assess the importance of measuring MAP kinase in human breast cancer tissue.

Role of Reactive Oxygen Species in Cancer

Reactive oxygen species (ROS) are oxygen containing chemical species with reactive chemical properties generated inside the cell as the consequence of respiration and enzymatic activities. ROS include O_2^-derived free radicals such as superoxide anion radical (O_2^-) and the hydroxyl radical (·OH), which contain an unpaired electron, and nonradical derivatives of O_2 such as hydrogen peroxide (H_2O_2) [136, 137]. Chemical and physical agents that produce ROS can induce and/or modulate the multistep carcinogenesis process [138]. Recent studies have shown an important role for reactive oxygen species in tumor development [139, 140]. Oxidative stress may cause DNA, protein, and/or lipid damage, leading to changes in chromosome instability, genetic mutation, and/or modulation of cell growth that may result in cancer.

ROS can be produced from endogenous sources, such as from mitochondria, peroxisomes, and inflammatory cell activation; and exogenous sources, including environmental agents, pharmaceuticals, and industrial chemicals [138]. Various environmental agents like, chlorinated compounds, radiation, metal ions, barbiturates, phorbol esters, acrylonitrile, and some peroxisome-proliferating compounds have been shown to induce oxidative stress and damage in vitro and in vivo studies [141]. Mutation studies have suggested that chronic oxidative stress is associated with carcinogenesis. Oxidative DNA damage is a major source of the mutation load in living organisms, with more than one hundred oxidative DNA adducts (purine, pyrimidine, and the deoxyribose backbone) having been identified [142-145]. The estimated frequency of oxidative DNA damage in human cells is 10^4 lesions/ cell/ day [142, 146]. High dose exposure to carcinogens has been shown to induce ROS in some studies [147], but the effect of low dose environmental carcinogens in ROS production still needs to be validated. Also how ERK pathway and ROS roles in carcinogenesis need to be investigated.

Stem Cells and Cancer

According to the most widely accepted views, stem cells are the cells that can "***self-renew***" to produce more cells and to generate daughter cells that can differentiate to several lineages to form all cell types found in a tissue. The stem cells might undergo a special kind of cell division where each stem cell divides into two asymmetric cells, one of which is differentiated to various types of cells and the other retains the capability of being the stem cells, renewing the stem cell pool of the body by further asymmetric division [148, 149]. In this process the number of stem cells remains constant. The other defining property of stem cells is "***potency***" to different types of specialized cells. Stem cells can either be totipotent or pluripotent - to be able to give rise to any mature cell type, although multipotent or unipotent progenitor cells are sometimes also referred to as stem cells.

Types of stem cells

Two broad types of mammalian stem cells are:

1. **Embryonic stem cells**: Embryonic stem cells (ES cells) are stem cells derived from the inner cell mass of an early stage embryo known as a blastocyst [150].
2. **Adult stem cells**: Adult stem cells are undifferentiated cells, found throughout the body after embryonic development that multiply by cell division to replenish dying cells and regenerate damaged tissues. They are of various types depending on source organ:
   (a) Haematopoietic stem cells are found in the bone marrow and give rise to all the blood cell types [151].

(b) Mammary stem cells provide the source of cells for growth of the mammary gland during puberty and gestation and play an important role in carcinogenesis of the breast [152].

(c) Mesenchymal stem cells (MSCs) are of stromal origin and may differentiate into a variety of tissues. MSCs have been isolated from placenta, adipose tissue, lung, bone marrow and blood and Wharton's jelly from the umbilical cord [153].

(d) Neural stem cells are commonly cultured in vitro as so called neurospheres- floating heterogeneous aggregates of cells, containing a large proportion of stem cells [154].

(e) Olfactory adult stem cells have been successfully harvested from the human olfactory mucosa cells, which are found in the lining of the nose and are involved in the sense of smell [155].

(f) Dental pulp derived stem cells: Multipotent stem cells known as SHED cells (Stem cells Harvested from Exfoliated Deciduous teeth), have been shown to have the same cellular markers and differential abilities of mesenchymal stem cells.

(g) Testicular cells are multipotent stem cells with a claimed equivalency to embryonic stem cells have been derived from spermatogonial progenitor cells found in the testicles.

Cancer Cell

Cancer develops from normal cells that have gained the ability to aberrantly proliferate and lost the ability of growth check points [113]. Some key characteristics of cancer cells are:

Uncontrolled growth: The cancer cells divide in an uncontrolled manner and often are called ‘immortal’.

Override cellular signals: The cancer cells escape the signaling that control and organizes the growth and differentiation of normal cells.

Contact inhibition: They can grow as solid mass, rather than layers.

Undifferentiated: Cancers are mass of undifferentiated cells, which do not form any tissue, just divide uncontrollably.

Models for cancer progression:

Stochastic model predicts that every cancer cell has the potential to form a new tumor, but entry into the cell cycle is a stochastic event that occurs with low probability. The stochastic model postulates that the processes of self-renewal versus differentiation in single cells within a population occur randomly and are governed by probabilities. Accordingly, every tumor cell will have a low but equal probability of proliferating extensively and thus the potential to behave as a stem cell (**Figure 1.7a**). Importantly, only those cells that retain self-renewal capacity would have the ability to sustain neoplastic growth [156-158]. Under the assumptions that all cancer cells have similar potential to grow tumors and that tumors are usually clonal in origin, it can be expected that even a few cancer cells would be able to grow new tumors

Stem cell model proposes the existence of distinct classes of cells within a tumor, each with different capacities for self-renewal and proliferation. Consequently, only a small definable subset of cells will consistently have the capacity to initiate tumor growth and reproduce the hierarchy of cell types that comprise the tumor [157, 159, 160] (**Figure 1.7b**).

Cancer Stem Cell

More and more recent studies are strengthening the importance and role of stem cells in cancer progression (**Figure 1.8**).

Evidences for the cancer stem cell hypothesis:

1. Most of the mutations leading to cancer affect cellular machinery that controls cell division, DNA damage, and signal transduction pathways. Stem cells may be preferential targets of initial oncogenic mutations because in most tissues in which cancer originates they are the only long-lived populations and are therefore exposed to more genotoxic stresses than their shorter-lived, differentiated progeny [148, 156].
2. Most tumors arise from a single cell, but not all the cells within a tumor are identical. This concept is also known as tumor heterogeneity [161].
3. A large number of cancer cells were required to grow a tumor. These observations were seemingly at odds with the traditional stochastic model of cancer development [148].
4. Both recent and traditional therapies suggest that in most cases tumor always re grow, no matter how often the tumor mass is reduced [148].
5. Stem cells often persist for long periods of time, instead of dying after short periods of time like many mature cells in highly proliferative tissues. This means that there is a much greater opportunity for mutations to accumulate in individual stem cells than in most mature cell types [148, 161]

Breast Cancer and Mammary Stem Cells

The mammary gland in humans and other mammals is a dynamic organ that undergoes significant developmental changes during pregnancy, lactation, and involution.

The basic components of a mature mammary gland are the alveoli (hollow cavities) lined with milk-secreting cuboidal cells and surrounded by myoepithelial cells. These alveoli join up to form groups known as lobules, and each lobule has a lactiferous duct that drains into openings in the nipple. During pregnancy and lactation lobulo-acinar structure containing milk secretion alveolar cells are extensively formed (**Figure 1.9**). After lactation the organ goes through massive apoptosis and tissue remodeling to go back to normal form [162, 163]. So the mammary gland is a dynamic tissue and it should have some progenitor cells to sustain the proliferation during pregnancies.

Decades ago, Daniel et al [164] showed that complete mammary gland can be generated from random epithelial fragments. Recently, Shackleton et al [165] have shown that entire mammary gland can be generated using a single mammary "stem cell". So it is beyond doubt now that mammary gland is a source of adult stem cells in the body.

The isolation and characterization of mammary stem cells have been hindered because of two reasons, firstly, the scarcity of suitable markers and secondly, lack of suitable systems to maintain the stem cells in undifferentiated state [161].

Pioneering work by Al-Hajj et al [159], lead to identification of tumorigenic mammary stem cell markers. Cells expressing $CD44^{+}$ $CD24^{-/low}$ $Lineage^{-}$ have been shown to have ability to form complete tumor when injected into mice at low numbers like 100. Major advancement in culturing neural stem cell was achieved when they were grown as anchorage independent colonies in suspensions under low serum conditions, creating spheroids called *neurospheres*

[166]. A similar approach was used to grow mammary gland stem cells in suspensions as well, and these non-adherent spherical colonies were called *"Mammospheres"* [167].

Identification and Isolation of Mammary Stem Cells

With the recent advancement in stem cell research, it is now possible to identify and isolate mammary stem cells at better efficiency than before. There are various markers and characteristics that make them different from the surrounding differentiated cells.

Mammospheres

It is know that normal epithelial cells are anchorage dependent and they undergo anoikis, a special type of apoptosis that occurs when the cells are detached from extra cellular matrix, when grown in substratum-less conditions [168]. Undifferentiated multi-potent population of cells can be grown in suspension as mammospheres [167]. The *mammospheres* are enriched in stem cells or progenitor cells. After several passaging the number of stem cells if highly enriched, after four passages the mammosphere consists mostly of stem cells.

ALDH Activity

Ginestier et al [169] showed that normal and cancer human mammary epithelial cells with increased aldehyde dehydrogenase activity (ALDH) has stem/progenitor properties. These cells contain the subpopulation of normal breast epithelium with the broadest lineage differentiation potential and greatest growth capacity in a xenotransplant model. Aldehyde dehydrogenase is a polymorphic enzyme responsible for the oxidation of aldehydes to carboxylic

acids. These findings offer an important new tool for the study of normal and malignant breast stem cells and facilitate the clinical application of stem cell concepts [169]

Immunostaining

Like the other type of adult stem cells, mammary stem cells also have been identified using cell surface markers. Mammary SCA-1 (Stem Cell Antigen-1) has been used to identify the stem cells [170]. The immune-staining and cell sorting using surface markers is a very widely used technique to enrich the stem cell population. Breast cancer stem cells have also been identified with cell surface markers, $CD44^{+}$ $CD24^{-/low}$ $Lineage^{-}$ which are seen to be over expressed in breast tumor cells [159].

Extensive self-renewal and multi lineage differentiation

Stem cells can generate the entire repertoire of cell types found in a tissue. The stem cells derived from the mammospheres have the capacity to self-renew for extended number of times. Also the stem cells can differentiate into various types of cells; when they are grown on collagen substratum under differentiating conditions, like in 2D culture systems, matrigel or in presence of prolactin. Under these conditions the stem cells differentiate in to alveolar, acinar or ductal structures [161].

Dye exclusion ability

Stem cells, including neural, hematopoietic and mammary, have a characteristic property to exclude dyes such as rhodamine and Hoechst [171] as a result of increased expression of membrane transporter proteins, such as P-glycoproteins or BCRP (breast cancer resistance proteins). This unique dye exclusion property is because of ATP-binding cassette family of

transporter proteins, which pump outs the dye. This population of cells which has dye exclusion property is called “Side population” and it is generally enriched in stem cell surface markers.

Label retention ability

Several studies have shown that a sub-set of cells have the capacity to retain the label like BrdU or H^3-tymidine, these are designated as label retaining cells (LCR). Various studies have shown that very few of these LCR cells express differentiation markers and suggest that they are in less differentiated state and may be progenitor cells [170]. BrdU is incorporated in the DNA and gets diluted as the cells divides and is lost after certain number of divisions, long term maintenance of the label shows that the cells have a low proliferation rate that helps them to survive for much longer time than normal cells.

Epithelial to Mesenchymal Transition

The epithelial-mesenchymal transition (EMT) is an orchestrated series of events in which cell-cell and cell-extracellular matrix (ECM) interactions are altered to release epithelial cells from the surrounding tissue, the cytoskeleton is reorganized to confer the ability to move through a three-dimensional ECM, and a new transcriptional program is induced to maintain the mesenchymal phenotype [172]. For example, during early embryonic development, the mesoderm generated by EMTs develops into multiple tissue types, and later in development, mesodermal cells generate epithelial organs, such as the kidney and ovary, via Mesenchymal to Epithelial transition (MET) [173, 174]. Turning an epithelial cell into a mesenchymal cell requires alterations in morphology, cellular architecture, adhesion, and migration capacity. Through EMT, the polarized epithelial cell, which normally interacts with basement membrane via its basal surface, undergoes multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components [175] Molecular markers associated with EMT include increased expression of N-cadherin and vimentin, nuclear localization of β-catenin, and increased production of the transcription factors such as Snail1 (Snail), Snail2 (Slug), Twist, EF1/ZEB1, SIP1/ZEB2, and/or E47 that inhibit E-cadherin production. Phenotypic markers for an EMT include an increased capacity for migration and three-dimensional invasion, as well as resistance to anoikis/apoptosis [176] (**Figure1.10**). A summary of common EMT markers is listed in **Table 1.1** [176].

Induction of EMT has been shown to be highly tissue- and cell type-specific [177], because factors that induce EMT under some conditions and locations can have quite different effects in others [178]. Some of the cytoplasmic signal transduction pathways are fairly well

defined - for example, the activation and nuclear translocation of SMAD proteins following association of TGF-β with its cell surface receptors [179]. In other cases, activation of EMT involves more pleiotropic signals, as in the case of reactive oxygen species (ROS) produced in response to exposure to matrix metalloproteinases (MMPs) [172]. Several other oncogenic pathways like, peptide growth factors, Src, Ras, Ets, integrin, Wnt/beta-catenin and Notch may induce EMT [180]. In particular, Ras-MAPK has been shown to activate two related transcription factors known as Snail and Slug. Both of these proteins are transcriptional repressors of E-cadherin and their expression induces EMT [181]. Recently, some studies have shown activation of the phosphatidylinositol 3' kinase (PI3K)/AKT axis is emerging as a central feature of EMT [182] (**Figure 1.11**).

EMT and Cancer Stem Cell

During cancer progression, some cells within the primary tumor may reactivate the latent embryonic program of EMT. Through EMT, transformed epithelial cells can acquire the mesenchymal traits that seem to facilitate metastasis but, there is accumulating evidence that EMT and mesenchymal-related gene expression are associated with aggressive breast cancer subtypes and poor clinical outcome in breast cancer patients. EMT along with play a key role in normal embryo developmental processes has also been shown to play role during cancer invasion and metastasis [174]. Also, recently EMT has shown to generate cells with stem cells properties [174]. Developmental hierarchy has been demonstrated in breast tissue where, stem cells give rise to both the outer myoepithelial layer and the inner layer of luminal epithelial cells, which

define the bilayered architecture of the branching mammary gland [183-185]. Normal human mammary tissue contains a subpopulation of $EpCam^{high}/CD49f^{high}$ stem cells that give rise to mammospheres and to bilayered, branching structures in 3D culture [172, 185]. Recently, Mani et al reported that the induction of an EMT in immortalized human mammary epithelial cells (HMLEs) results in the acquisition of mesenchymal traits and in the expression of stem-cell markers [174]. Furthermore, they showed that these cells have an increased ability to form mammospheres, a property associated with mammary epithelial stem cells. Also, stem cell-like cells isolated from HMLE cultures form mammospheres and express markers similar to those of HMLEs that have undergone an EMT. Further they showed that stem-like cells isolated either from mouse or human mammary glands or mammary carcinomas express EMT markers and transformed human mammary epithelial cells that have undergone an EMT form mammospheres, soft agar colonies, and tumors more efficiently. These findings illustrate a direct link between the EMT and the gain of epithelial stem cell properties [174].

Presently only a very few studies have shown the importance of environmental carcinogens in process of EMT [186-188] and generation of cancer stem cells [189] , therefore more studies are needed to address this question. In the past decade the importance of cancer stem cells in cancer severity and recurrence after chemotherapy has been a highly investigated area. The increasing need to identify and target the cancer stem cell is a fast growing research area; therefore it is important to identify the origin and role of cancer stem cell during the precancerous stages of cancer progression. This will help to target and/or prevent the formation of cancer stem cell in the beginning and might be a powerful tool in reducing the risk of cancer relapse in patients.

Using the chronic precancerous model we want to study the stem-like cell and EMT related properties and markers induced by chronic exposure to environmental carcinogenesis that lead to the transition of normal cells to pre malignant to malignant cells.

Dietary Cancer Prevention

Wide geographical variation in cancer incidence and mortality rates is thought to be due to difference in lifestyle and environmental factors, including diet [190, 191]. Migrant studies comparing individuals who move from countries with low rates to those with high rates, and dynamic studies that link changes within countries to environmental or dietary change in those countries have suggested that diet and other lifestyle factors substantially influence cancer risk [192, 193]. Doll and Peto [109] suggested that dietary factors might contribute to an estimated one-third of all cancer deaths; however, this estimate can vary from 10% to 70% [194]. According to WHO at least 30–40% of all cancer deaths are preventable [195]. This means, that of the 7.4 million cancer deaths (or 13% of the total number of deaths worldwide) in 2004, 2.2–2.9 million could have been prevented [196]. More than 200 studies have examined the relationship between fruit and vegetable intake and cancers of the various organs, including, lung, colon, breast, cervix, esophagus, oral cavity, stomach, bladder and pancreas [197].

Natural dietary agents including fruits, vegetables, and spices have drawn a great deal of attention from both the scientific community and the general public owing to their ability to suppress cancers [198]. The questions that remain to be answered are which component of these dietary agents is responsible for the anti-cancer effects and what is the mechanism by which they suppress cancer. Fruits and vegetables are excellent sources of fiber, vitamins, and minerals, but they also contain components like flavonoids, polyphenols, terpenes, alkaloids, and phenolics that may provide substantial health benefits beyond basic nutrition. Research over the last decade has shown that several micronutrients in fruits and vegetables reduce cancer [198]. Given the important role of diet in cancer prevention, a better understanding of the preventive approaches needs to be taken seriously. Early detection, treatment and prevention would seem to constitute

the complete strategy for cancer control (**Figure 1.12**). Effective anticancer strategies are therefore crucial and should meet specific criteria in order to succeed. These strategies must be practical and focus on high-risk populations, such as smoker [196].

Cancer preventive substances in vegetables and fruit

Many potentially anti-carcinogenic substances have been identified in vegetables and fruit, some of these substances are listed in **Table 1.2**. Some of these compounds are characteristic of particular classes of vegetables and fruit [199]. For example, cruciferous vegetables are unique in their high content of dithiolthiones and isothiocyanates; these are organosulfur compounds that have been shown to increase the activity of enzymes involved in the detoxification of carcinogens and other foreign compounds [200]. The allium vegetable family includes onions, garlic, scallions, leeks, and chives and is notable for its content of compounds such as diallyl sulfide and allyl methyl trisulfide, which have been shown to induce enzymatic detoxification systems [201]. Soybeans contains isoflavones like, genistein which has been shown to inhibit the activity of tyrosine kinase, an enzyme involved in the transmission of signals from cellular growth factor receptors that is expressed at high levels in transformed cells [202]. Citrus fruit is known for its high content of vitamin C, which, as an antioxidant, may protect cell membranes and DNA from oxidative damage. Vitamin C may further help prevent cancer via its ability to scavenge and reduce nitrite, thereby reducing substrate for the formation of nitrosamines [203]. Green leafy vegetables contain lutein, a carotenoid and xanthophyll pigment which is an antioxidant and may protect against cancer via its ability to block damage by free radicals [204]. Orange vegetables, such as carrots, sweet potatoes, winter squash, and pumpkin, are relatively rich sources of beta carotene, as are some fruits, including papaya,

mango, and cantaloupe. Beta carotene, like other carotenoids, is an antioxidant; through this function it may protect against free radical damage [205].

Many other potentially anticarcinogenic substances are not limited to one type of vegetable or fruit but are more widespread. Flavonoids, such as quercetin and kaempferol, are polyphenolic antioxidants that occur in vegetables and fruit (tea and wine are also important sources) [206]. In addition to being antioxidants, flavonoids may defend cells against carcinogens via their ability to increase the pump-mediated efflux of certain carcinogens from cells [207] or via induction of detoxification enzymes [208]. Fruit, vegetables, and legumes are major sources of dietary fiber, which has been widely hypothesized to be protective against colon cancer [209].

Therefore, various types of vegetables and fruits have different active compounds that can help prevent cancer through multiple mechanisms, so a balanced healthy diet is recommended for cancer prevention [199]. In this study we have examined the importance of two dietary compounds, green tea and grape seed, in prevention of chronic breast cancer carcinogenesis.

Green Tea

Green tea is made solely with the leaves of Camellia sinensis that have undergone minimal oxidation during processing. It is the most globally consumed beverage in the world. [210, 211]. It is estimated that about 2.5 million tons of tea leaves are produced throughout the world each year with 20% produced as green tea, which is mainly consumed in Asia, some parts

of North Africa, the United States, and Europe [211]. It contains characteristic polyphenolic compounds, (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epicatechin (EC), the usual composition is 10–15% EGCG, 6–10% EGC, 2–3% ECG, and 2% EC [212].

A growing body of evidence from laboratory animal studies demonstrates that tea consumption has an inhibitory effect on carcinogenesis at various organ sites. For example, oral administration of tea infusion can inhibit the development of experimental rodent skin tumors,[213] growth of implanted tumor cells,[214] invasion and metastasis of malignant tumors, [215, 216] and angiogenesis [217, 218]. At present, epidemiological studies have not yielded conclusive evidence of the protective effect of tea consumption against the development of human cancers [219-221]. However, limited epidemiological studies suggest that people drinking more cups of tea regularly have a lower risk of prostate and breast cancer [222-224].

Animal studies show that green tea catechin extract (GTC) is able to suppress rat mammary carcinogenesis induced by 7,12-dimethylbenz[a]anthracene and N-methyl-Nnitrosourea [225, 226]. Laboratory studies also have shown that GTC possesses inhibitory and apoptotic activity in human breast cancer cells in cultures [227, 228]. Although controversial, epidemiological studies have examined the benefits of tea consumption for breast cancer prevention, and some evidence has indicated that green tea consumption may help prevent breast cancer recurrence in early stage cancers [229-231]. Thus, it is important to identify carcinogens whose induction of breast cell carcinogenesis can be intervened by green tea components to reveal targets for dietary prevention.

Epicatechin (EC)

Catechin possesses two benzene rings (called the A- and B-rings) and a dihydropyran heterocycle (the C-ring) with a hydroxyl group on carbon 3. Two of the isomers are in trans-configuration and are called catechin and the other two are in cis configuration and are called epicatechin (**Figure 1.13a**). Epicatechin is a flavanol commonly present in higher plants, its present in high content in certain food plants, such as *Vitis vinifera* (grape wine), *Camellia sinensis* (tea), and *Theobroma cacao* (cocoa). Mice studies have identified that when animal is given a single modest dose of epicatechin and then followed by induction of ischemic stroke by cutting off blood supply to the animals' brains they suffered significantly less brain damage than animals that had not been given the compound [232]. Epicatechin and its dimers have been shown to inhibit NADPH-oxidase and the subsequent superoxide production [233]. Also, epicatechin could act at different levels in signaling pathways involving protein kinases and phosphatases and some studies show its role in preventing gastrointestinal cancer [233].

Epicatechin-3-gallate (ECG)

Epicatechin gallate is a flavan-3-ol, a type of flavonoid, present in green tea (**Figure 1.13b**). ECG can interfere with multiple cell signaling pathways and has multiple targets within the cells, which are likely to interact together to reduce the risk of carcinogenesis. These mechanisms include (a) inhibition of phase 1 CYP enzymes, (b) induction of phase II detoxification and antioxidant enzymes, (c) anti-inflammatory efficacy (d) arrest of cell cycle progression, (e) regulation of pro-apoptotic properties and (f) mediation of metastasis processes [234-238]. Many of the anti-carcinogenic affects of ECG may be due to its direct and/or indirect interaction with numerous molecular targets [239] such as NAG-1, AP-1, 5α-reductase and

PDGF. Importantly, these growth inhibitions of ECG have been shown to sensitize cancer cells, but not in normal cells. ECG may also inhibit RNase A and MMPs enzymatic activity via chelating copper and zinc metals, which are important cofactors for angiogenesis and metastasis. Furthermore, structure function analysis revealed that the gallate moiety of ECG is important for mediating these inhibitory effects which these acts may enhance chemoprevention ability [238].

Epigallocatechin (EGC)

Epigallocatechin is a flavan-3-ol, a type of chemical compound including catechin, with the gallate residue being in an isomeric cis position (**Figure 1.13c**). The major sources of EGC are green tea, along with bananas, persimmon and pomegranate. ECG has shown to potentiate effect on high K+-induced contraction in isolated porcine coronary arteries without endothelium [240], suppressed growth of human melanoma cell line UACC-375 [241]. More studies are needed to identify the role of EGC in other type of cancer prevention.

Epigallocatechin-3-gallate (EGCG)

Epigallocatechin 3-gallate (EGCG), is the ester of epigallocatechin and gallic acid (**Figure 1.13d**). EGCG is the major component of the polyphenolic fraction of green tea. Along with other tea catechins, and polyphenols in general, it is an antioxidant that is thought to prevent tumorigenesis by protecting cellular components from oxidative damage via free radical scavenging. Increasing number of evidences indicate that EGCG can be beneficial in treating brain, prostate, cervical and bladder cancers [242-244]. EGCG has been shown to bind and inhibit the anti-apoptotic protein Bcl-xl [245], which has been implicated in both cancer cell and normal cell survival [246]. Indeed, a number of studies have demonstrated the free radical scavenging activites of EGCG, as well as its antimutagenic, antitumorigenic, anti-angiogenic,

antiproliferative, and/or pro-apoptotic effects on mammalian cells both in vitro and in vivo. Also, wide variety of research shows EGCG restrained carcinogenesis in a variety of tissues through inhibition of mitogen-activated protein kinases (MAPK), growth factor-related cell signaling, activation of activator protein 1 (AP-1) and nuclear factor-B (NF-kappaB), topoisomerase I, matrix metalloproteinases and other potential targets. Therefore, EGCG is a multipotent anticancer agent, which not only provides solid evidence to support the anticancer potential of green tea, but also offers new clues for discovering multiple-targeted anticancer drug [247].

Grape Seed

Grape seed extracts are industrial derivatives from whole grape (*Vitis vinifera*) seeds that have a great concentration of vitamin E, flavonoids, linoleic acid and polyphenols, including oligomeric proanthocyanidins which are recognized as antioxidants. Within the superfamily of polyphenolic compounds are proanthocyanidins, which are enriched in grape seeds and are oligomers and polymers (**Figure 1.14**) of the catechin family [248]. Grape seed proanthocyanidin extract (GSPE) has been shown to exhibit antioxidant and anticancer activities in both in vitro and in vivo models [249-252]; for example, it shows some diet-dependent, chemopreventive activity in a rat model with mammary cancer induced by 7,12-dimethylbenz[a]anthracene (DMBA) [252]. GSPEs have been shown to be potent antioxidants and free radical scavengers, being more effective than either ascorbic acid or vitamin E [253, 254]. Furthermore, GSPEs have been shown to have anticarcinogenic activity in different cancer like, skin, colorectal, prostate and breast cancers [255]. Thus scientific research shows

consumption of grapes and/or grape-related products in diets along with maintaining an active healthy lifestyle has both practical and translation potential in the fight against cancer and is thus beneficial to the general population [255]. Whether dietary GSPE is able to prevent the breast cell carcinogenesis associated with chronic exposures to environmental carcinogens needs to be studied.

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APPENDIX

Table 1.1

List of known EMT markers

Proteins that increase in abundance	**Proteins whose activity increases**
N-cadherin	ILK
Vimentin	GSK-3β
Fibronectin	Rho
Snail1 (Snail)	**Proteins that accumulate in the nucleus**
Snail2 (Slug)	β-catenin
Twist	Smad-2/3
Goosecoid	NF-κβ
FOXC2	Snail1 (Snail)
Sox10	Snail2 (Slug)
MMP-2	Twist
MMP-3	**In vitro functional markers**
MMP-9	Increased migration
Integrin αvβ6	Increased invasion
Proteins that decrease in abundance	Increased scattering
E-cadherin	Elongation of cell shape
Desmoplakin	Resistance to anoikis
Cytokeratin	

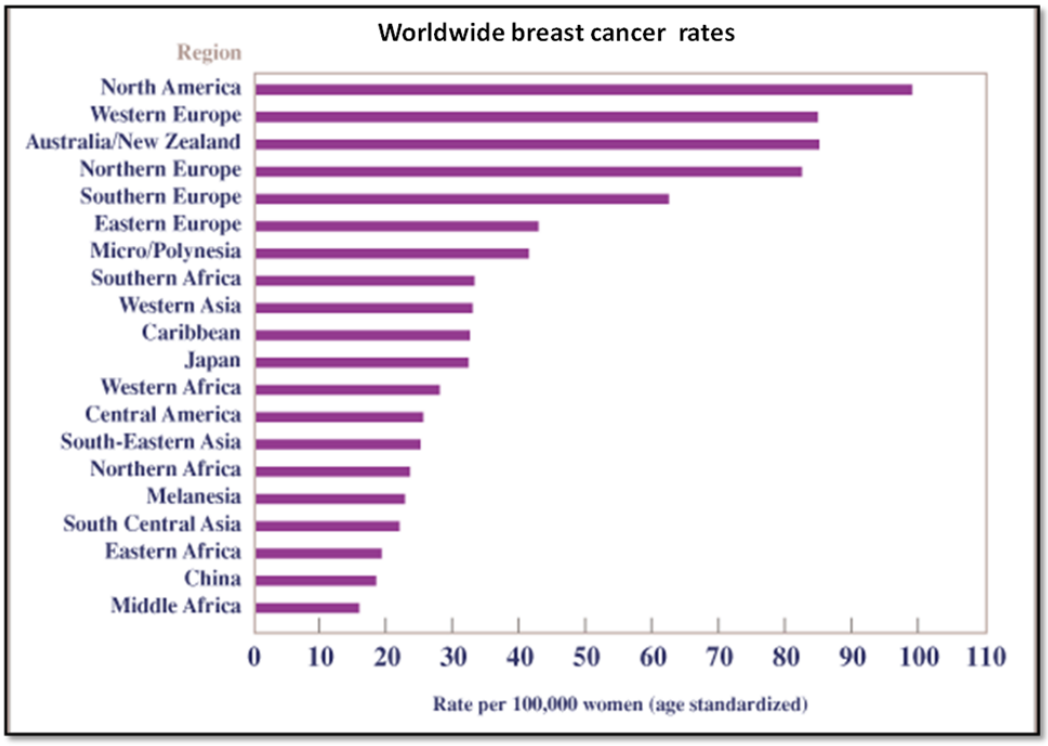
Modified from Journal of Cell Biology 2006 Mar 27;172(7):973-81.

The epithelial-mesenchymal transition: new insights in signaling, development, and disease.

Table 1.2

List of cancer preventive substances and their common sources

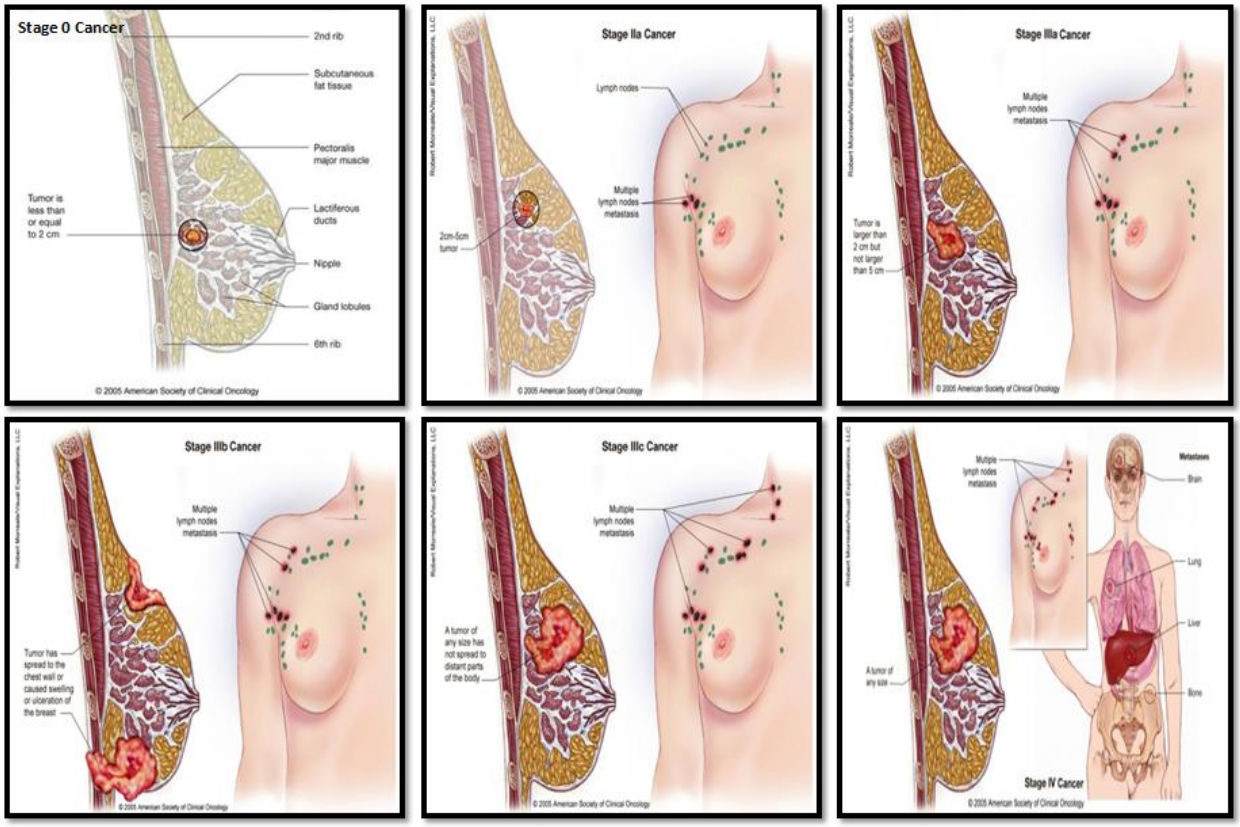
Preventive Compound	Source
Allium Compound	Onions, garlic, scallions, leeks, and chives
Carotenoids	Carrots, sweet potatoes, spinach, kale, collard greens, and tomatoes, all dark green leafy vegetables
Coumarins	Tonka bean, mullein, cassia, cinnamon, sweet clover
Dietary fiber	Soybeans and other legumes
Flavonoids	Green tea, wine, citrus fruits, dark chocolate
Folic acid	Leafy vegetables, cereals
Indole-3-carbinal	Broccoli, cabbage, cauliflower, brussels sprouts, collard greens and kale.
Isoflavones	Soybean, green bean, alfalfa sprout, cowpea
Isothiocyanate	Horseradish, mustard, radish, brussels sprouts, watercress, nasturtiums and capers.
Vitamin C	Citrus fruits, chilies, broccoli, strawberries



Modified from Susan G. Komen website (http://www.komennyc.org)

Figure 1.1

Worldwide Breast cancer incidence in 2007



Modified from American Society for Clinical Oncology (http://www.asco.org/)

Figure 1.2

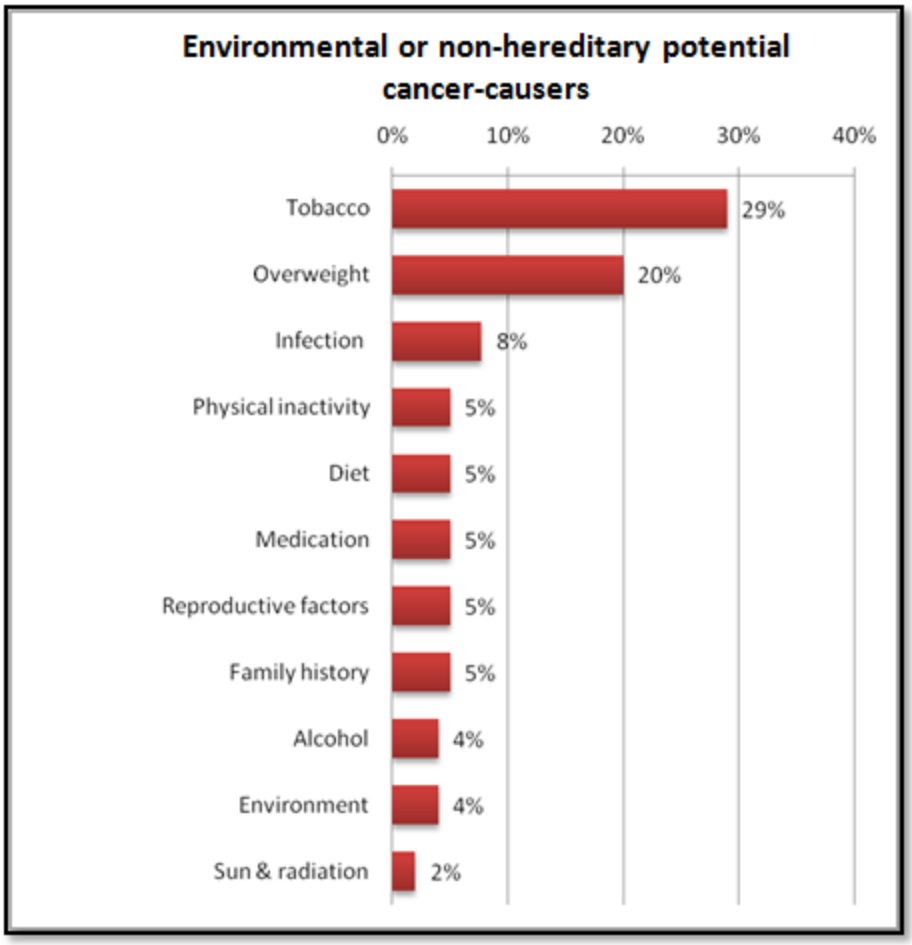
Stages of Breast Cancer as described by American Cancer Institute

Non-Modifiable Risk Factors	Modifiable Risk Factors
Gender	Radiation exposure
Age	Reproduction
Personal breast care	Breast-feeding
Family history	Hormone replacement therapy
Proliferative breast conditions	Oral contraceptives
Breast density	Body weight
Early menstruation	Physical activity
Late menopause	Alcohol / tobacco use

Modified from Breast Cancer Organization (http://www.breastcancer.org/)

Figure 1.3

Risk factors for breast cancer

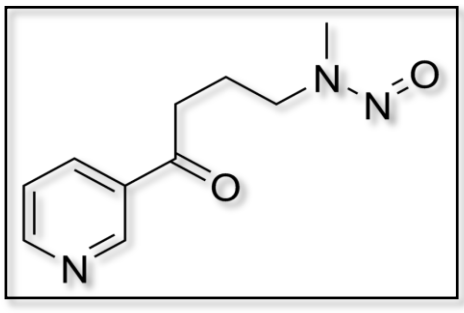


Adapted from Cancer News in Context (http://www.cancernewsincontext.org/)

Figure 1.4

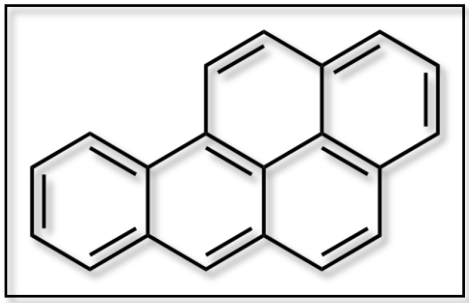
Environmental or non-hereditary potential cancer-causers

(A)



Adapted from Wikipedia

(B)



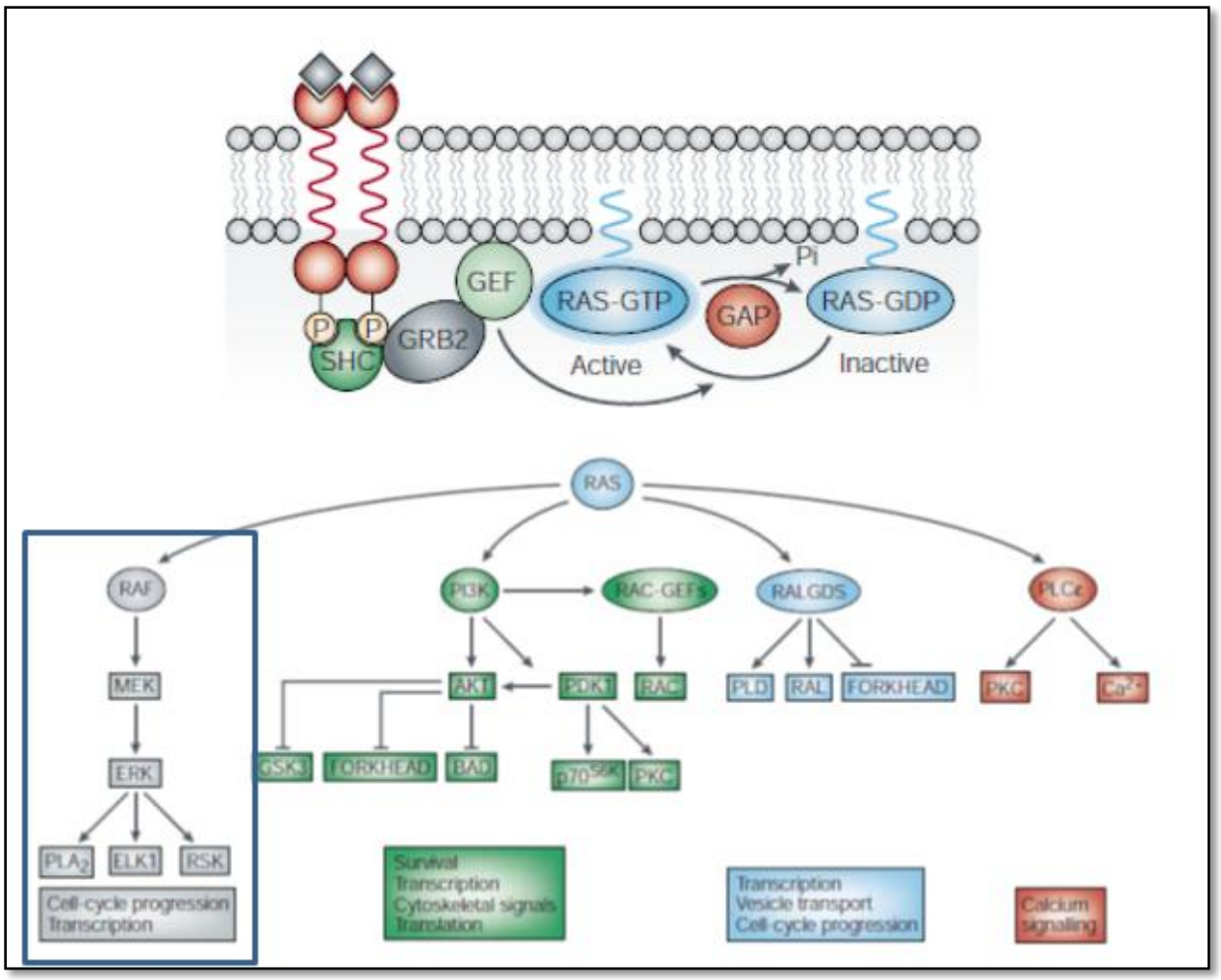
Adapted from Wikipedia

Figure 1.5

Chemical structure of carcinogens

(A) Chemical structure of 4-(methylnitrosamino)- 1-(3-pyridyl)-1-butanone

(B) Chemical structure of benzo[a]pyrene

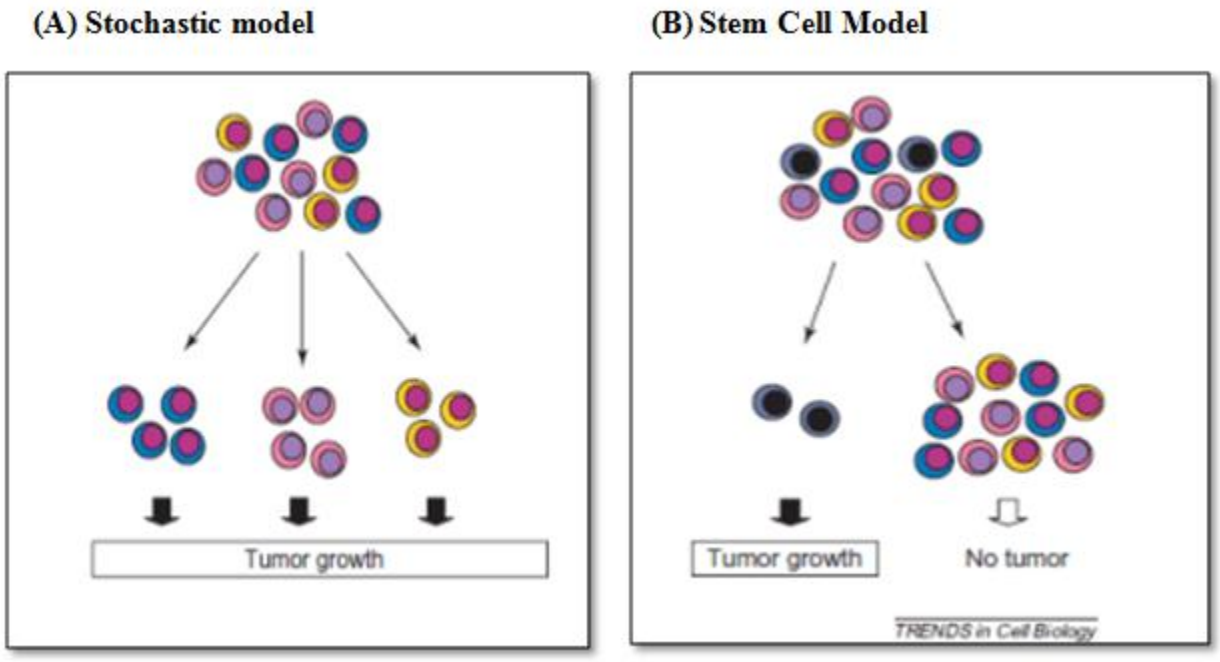


Modified from Nature Reviews Cancer. 2003 Jan;3(1):11-22.

Targeting RAS signalling pathways in cancer therapy.

Figure 1.6:

Ras activation and ERK pathway



Adapted from Trends Cellular Biology 2005 Sep;15(9):494-501.

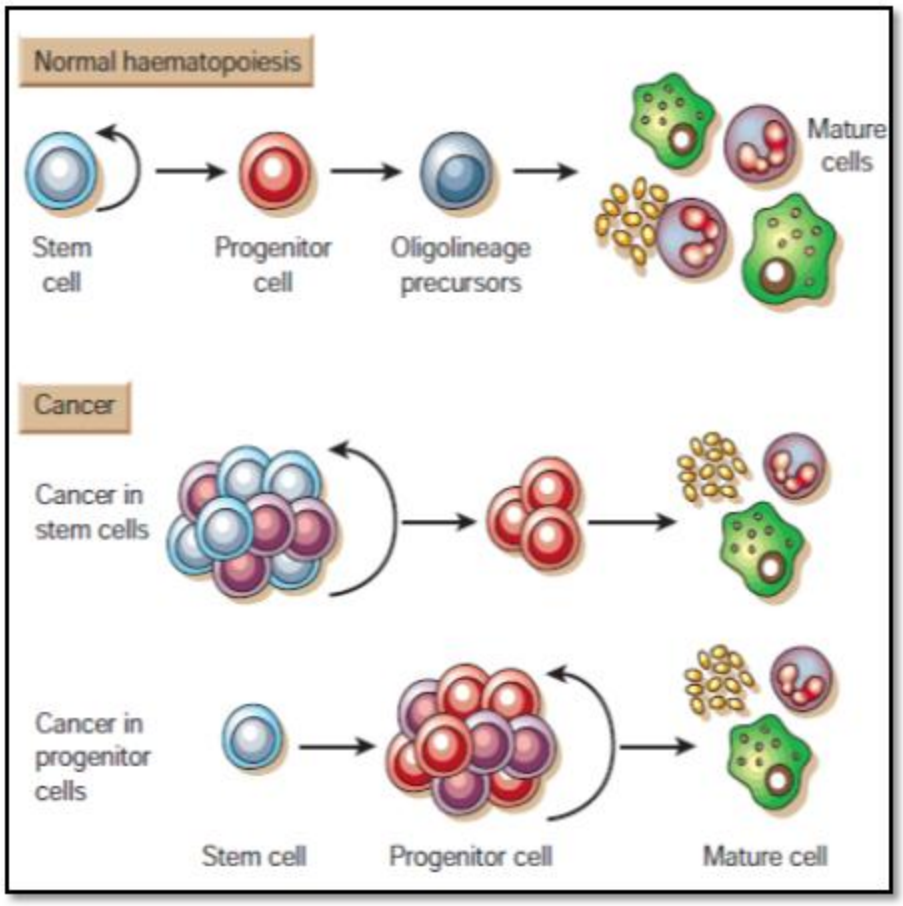
Cancer stem cells: lessons from leukemia.

Figure 1.7

Models of Cancer Progression

(A) Stochastic Model

(B) Stem cell Model

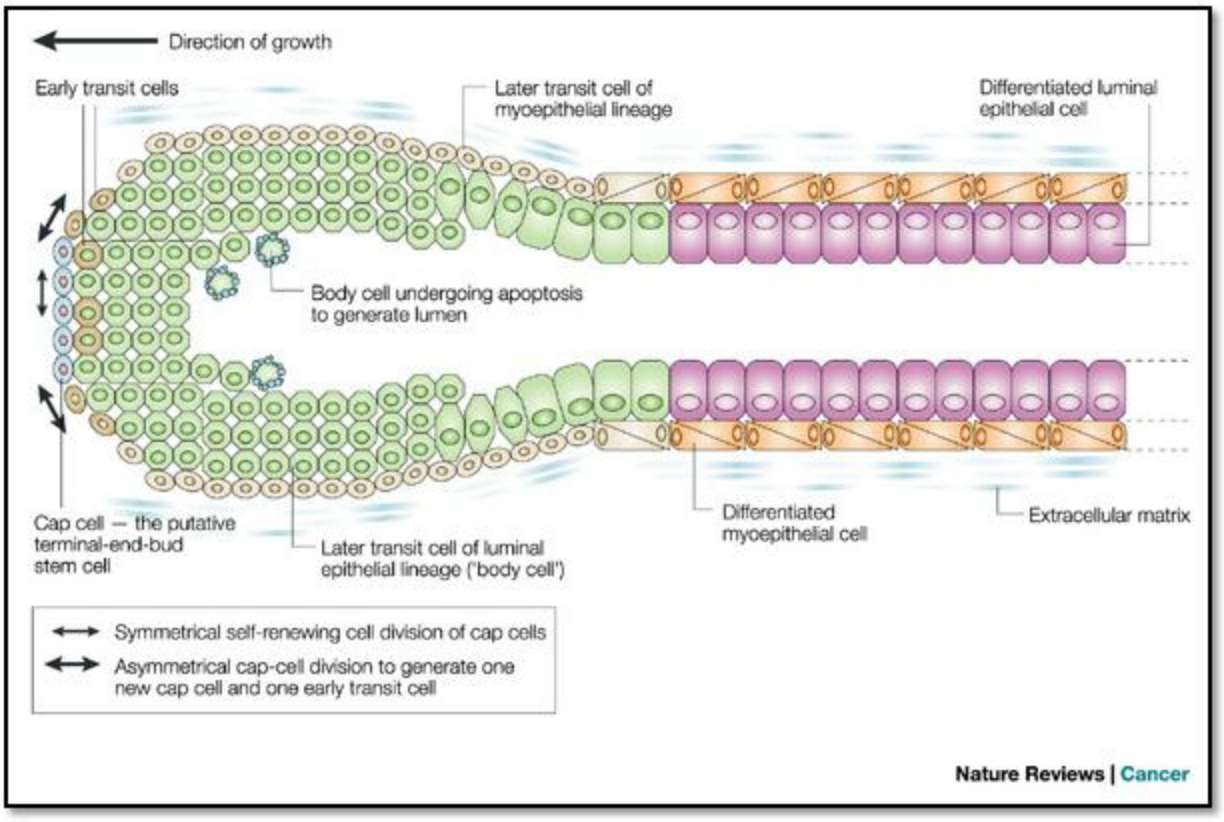


Adapted from Nature. 2001 Nov 1;414(6859):105-11.

Stem cells, cancer, and cancer stem cells.

Figure 1.8

Origin of cancer stem cells

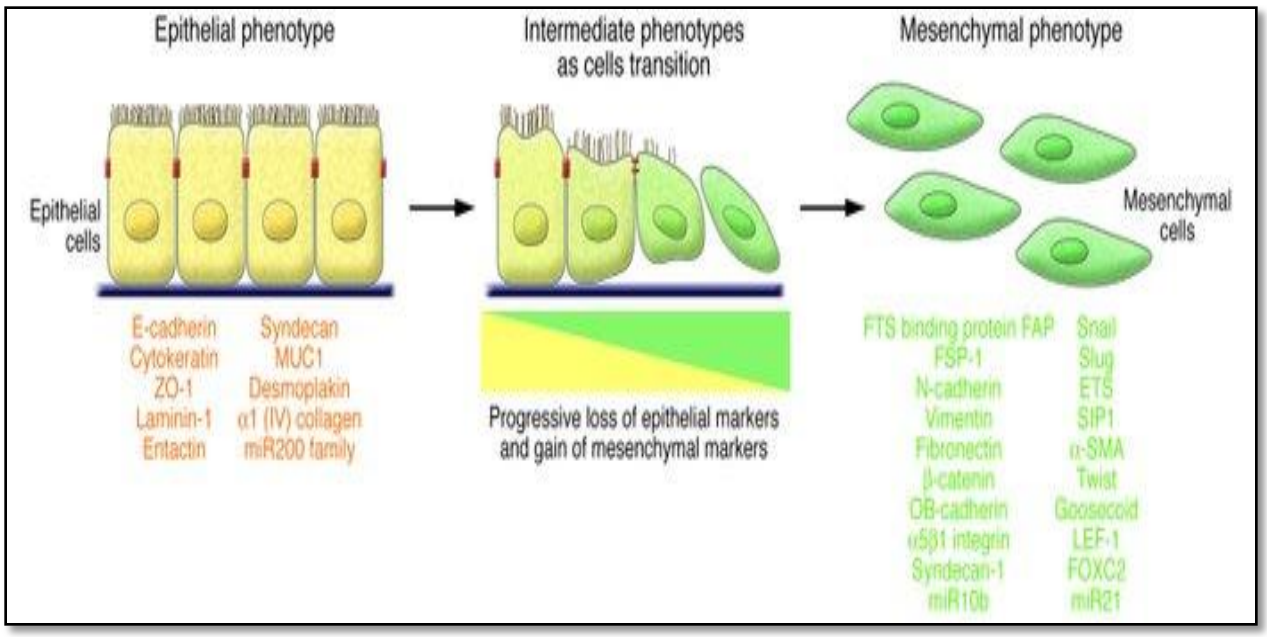


Adapted from Nature Review Cancer. 2003 Nov;3(11):832-44.

Stem cells and breast cancer: A field in transit.

Figure 1.9

Dynamicity of mammary gland

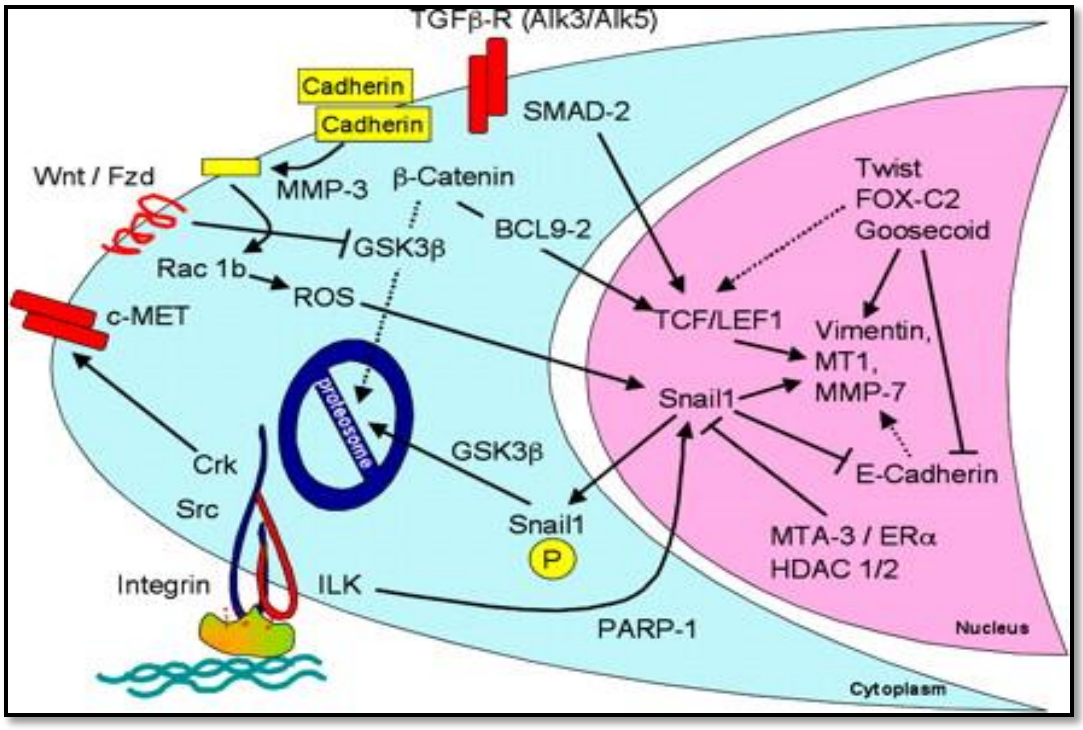


Modified from Journal of Clinical Investigation 2009 Jun;119(6):1420-8.

The basics of epithelial-mesenchymal transition.

Figure 1.10

Changes during Epithelial to Mesenchymal

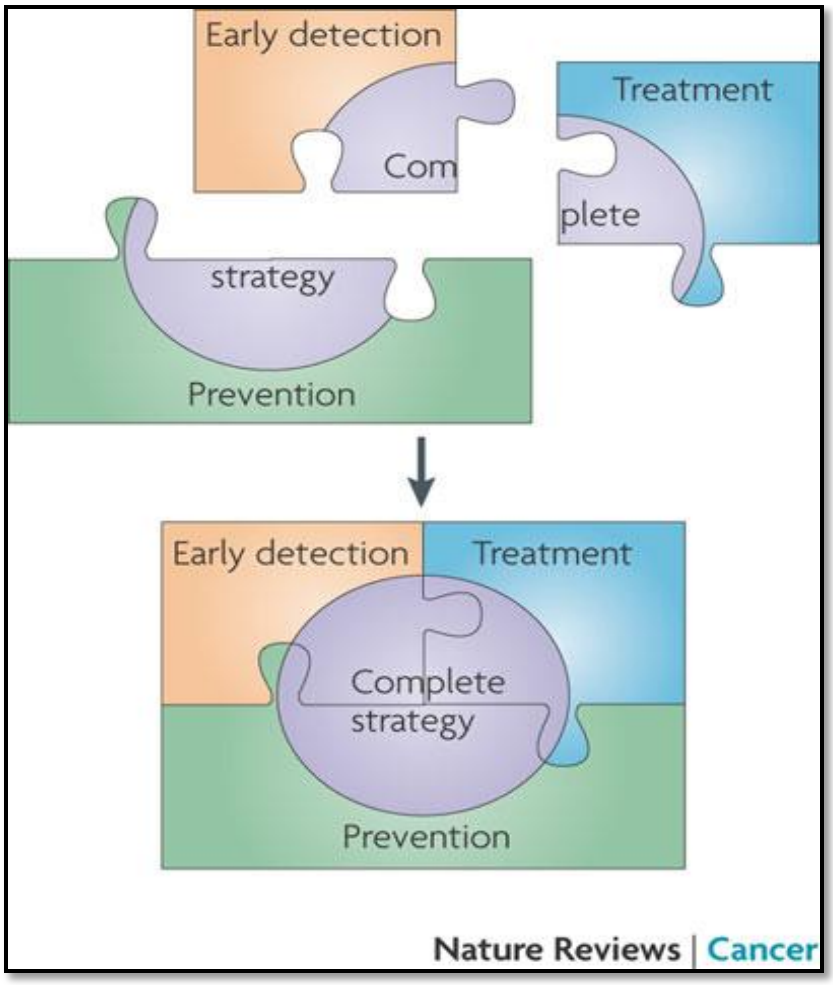


Modified from Journal of Cell Biology 2006 Mar 27;172(7):973-81.

The epithelial-mesenchymal transition: new insights in signaling, development, and disease.

Figure 1.11

Pathways activated during Epithelial to Mesenchymal Transition

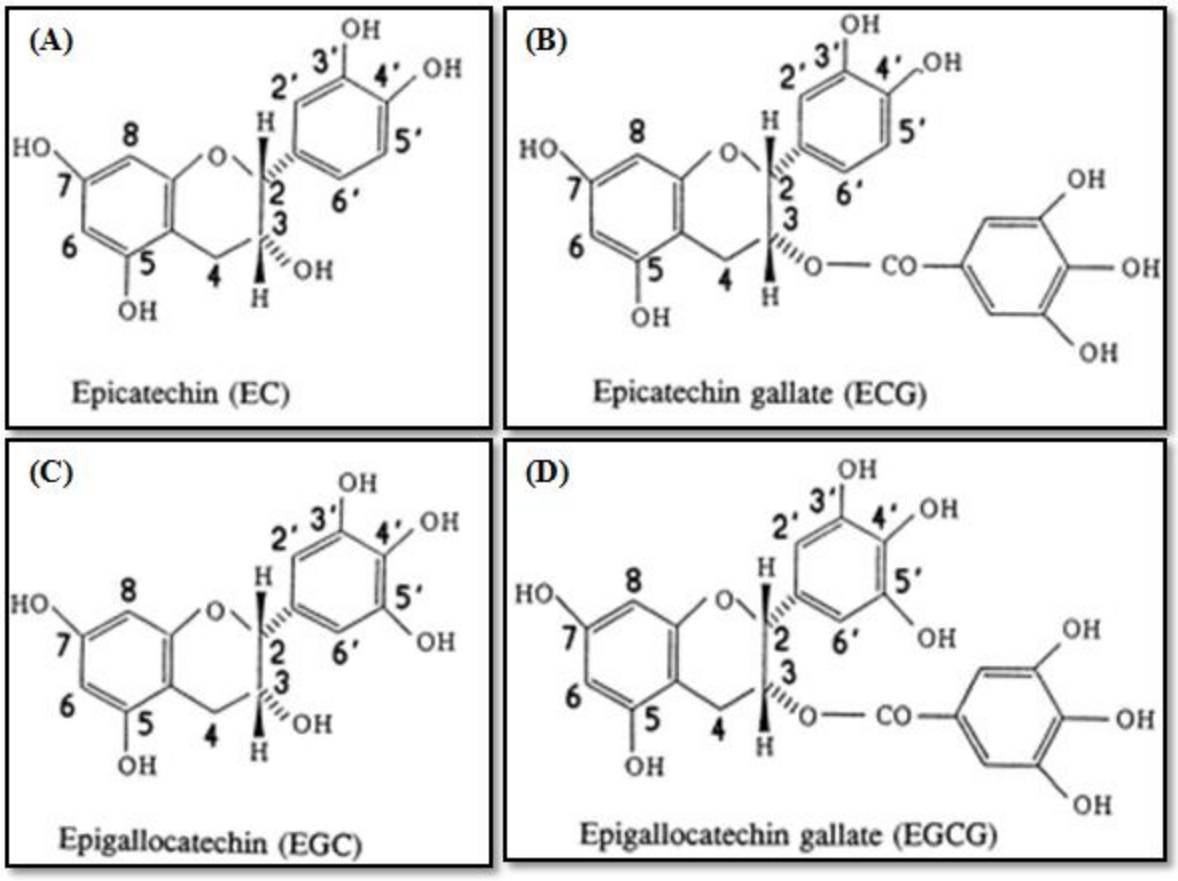


Adapted from Nature Reviews Cancer Jul;9(7):508-16

Cancer prevention research - then and now.

Figure 1.12

Complete strategy for eliminating cancer.



Adapted from Journal of Nutrition Jun;129(6):1094-101.

Jasmine green tea epicatechins are hypolipidemic in hamsters fed a high fat diet.

Figure 1.13

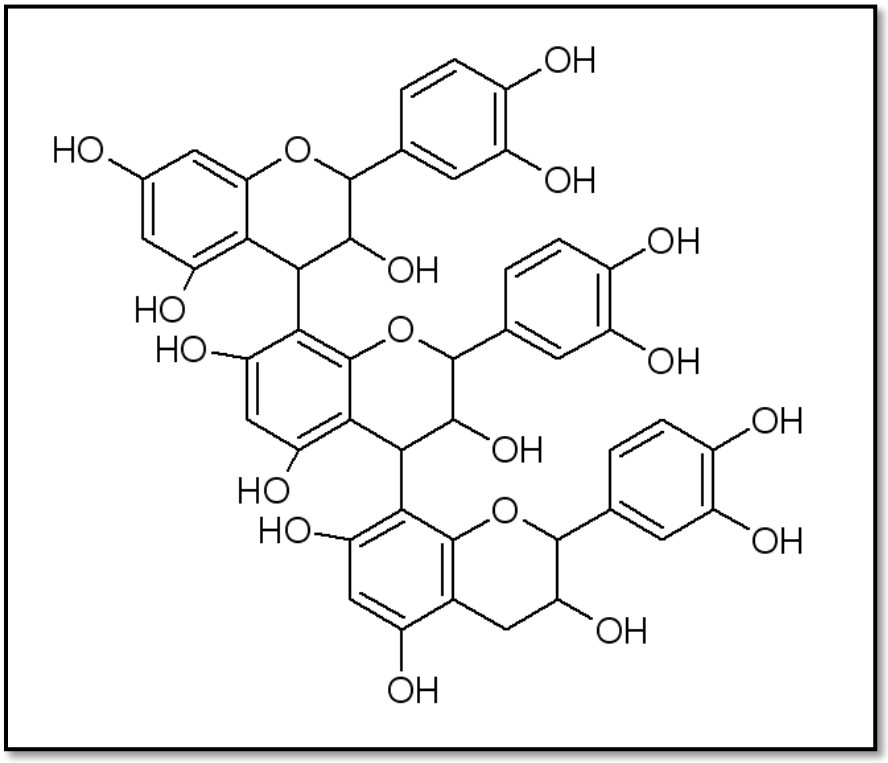
Chemical structure of Catechins

(A) Chemical structure of Epicatechin

(B) Chemical structure of Epicatechin-3-gallate

(C) Chemical structure of Epigallocatechin

(D) Chemical structure of Epigallocatechin-3-gallate



Adapted from Wikipedia

Figure 1.14

Chemical structure of Proanthocyanidin

PART –II

Green tea catechin extract in intervention of chronic breast cell carcinogenesis induced by environmental carcinogens

Research described in this chapter is slightly modified version of an article that is published in 2011 in Molecular Carcinogenesis by Kusum Rathore and Hwa-Chain Robert Wang

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In this paper “our” and “we” refers to me and co-authors. My contribution in the paper includes (1) Selection of the topic (2) Compiling and interpretation of the literature (3) Designing experiments (4) understanding the literature and interpretation of the results (5) providing comprehensive structure to the paper (6) Preparation of the graphs and figures (7) Writing and editing

Abstract

Sporadic breast cancers are mainly attributable to long-term exposure to environmental factors, via a multi-year, multi-step, and multi-path process of tumorigenesis involving cumulative genetic and epigenetic alterations in the chronic carcinogenesis of breast cells from a non-cancerous stage to precancerous and cancerous stages. Epidemiologic and experimental studies have suggested that green tea components may be used as preventive agents for breast cancer control. In our research, we have developed a cellular model that mimics breast cell carcinogenesis chronically induced by cumulative exposures to low doses of environmental carcinogens. In this study, we used our chronic carcinogenesis model as a target system to investigate the activity of green tea catechin extract (GTC) at non-cytotoxic levels in intervention of cellular carcinogenesis induced by cumulative exposures to pico-molar 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a]P). We identified that GTC, at a non-cytotoxic, physiologically-achievable concentration of 2.5 μg/mL, was effective in suppressing NNK- and B[a]P-induced cellular carcinogenesis, as measured by reduction of the acquired cancer-associated properties of reduced dependence on growth factors, anchorage-independent growth, increased cell mobility, and acinar-conformational disruption. We also detected that intervention of carcinogen-induced elevation of reactive oxygen species (ROS), increase of cell proliferation, activation of the ERK pathway, DNA damage, and changes in gene expression may account for the mechanisms of GTC's preventive activity. Thus, GTC may be used in dietary and chemoprevention of breast cell carcinogenesis associated with long-term exposure to low doses of environmental carcinogens.

Introduction

More than 70% of sporadic breast cancers are attributable to long-term exposure to environmental factors, such as chemical carcinogens; this chronic disease process involves accumulated genetic and epigenetic alterations to induce progressive carcinogenesis of breast cells from non-cancerous to precancerous and cancerous stages [1-4]. The current paradigm of experimental studies routinely uses high doses of carcinogens (micro- to milli-molar concentrations) to induce cancerous cells in cultures and tumors in animals as steps in evaluating the potency of carcinogens [1,3,5]. However, considering that long-term, chronic exposure to low doses of carcinogens is responsible for human breast cancer, a high-dose approach may not be a proper way to study environmental carcinogens in human breast cancer development.

We have been developing a cellular model to mimic chronic breast cell carcinogenesis occurring with accumulated exposures to low doses of environmental carcinogens, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and/or benzo[a]pyrene (B[a]P) [6-9]. NNK is a tobacco-specific carcinogen [10,11]. Although gastric administration of NNK into rats results in DNA-adduct formation in the mammary gland [12] and development of mammary tumors [13], NNK is not currently recognized as a mammary carcinogen. B[a]P is an environmental, dietary, and tobacco carcinogen [14-18]. Epidemiologic, animal, and cellular studies indicate that B[a]P may contribute to sporadic breast cancer development through its metabolites forming DNA-adducts causing DNA lesions [17-20]. However, the association between smoking and breast cancer is still controversial; some studies have indicated there is no influence of smoking on breast cancer incidence [21], and some studies have shown a correlation between them [22]. Recently, a study was conducted by four major Canadian agencies to address

this controversy, and the results indicate that active smoking and second-hand smoke increase breast cancer risk [23]. Another recent cohort study in the United States has also shown a connection between smoking and breast cancer in post-menopausal women [24]. Thus, the role of smoking in increasing the incidence of breast cancer should be taken seriously. Our model system has successfully revealed that NNK and B[a]P, at pico-molar ranges, like those detected in patients, are able to induce non-cancerous breast epithelial MCF10A cells to increasingly acquire cancer-associated properties via cumulative exposures [6-9].

The use of green tea to increase the body's antioxidant activity is becoming increasingly popular in the Western world [25]. Animal studies show that green tea catechin extract (GTC) is able to suppress rat mammary carcinogenesis induced by 7,12-dimethylbenz[a]anthracene and N-methyl-N-nitrosourea [26,27]. Laboratory studies also have shown that GTC possesses inhibitory and apoptotic activity in human breast cancer cells in cultures [28,29]. Although the are controversial, epidemiological studies have examined the benefits of tea consumption for breast cancer prevention, and some evidence has indicated that green tea consumption may help prevent breast cancer recurrence in early stage cancers [30-32]. Thus, it is important to identify carcinogens whose induction of breast cell carcinogenesis can be intervened by green tea components to reveal targets for dietary prevention. Using our cellular model as a target, we have tested the ability of GTC at a non-cytotoxic concentration of 40 μg/mL to suppress, by more than 50%, the acquisition of cancer-associated properties induced by cumulative exposures to 100 pmol/L of B[a]P [7]. Hence, it is important to pursue an extended study to reveal the effectiveness and mechanisms of GTC in intervention of chronic breast cell carcinogenesis caused by NNK and B[a]P for developing targeted intervention of breast cell carcinogenesis.

In this study, we used our cellular model as a target and investigated the minimal concentration of GTC required for intervention of NNK- and B[a]P-induced cellular carcinogenesis. We also investigated the mechanisms for GTC in counteracting the activity of NNK and B[a]P in inducing cellular, biochemical, and molecular changes on initiation of cellular carcinogenesis.

Materials and Methods

Cell Cultures and Reagents

MCF10A (American Type Culture Collection [ATCC], Rockville, MD) and derived cells were maintained in complete (CM) medium (1:1 mixture of DMEM and Ham's F12, supplemented with mitogenic additives including 100 ng/ml cholera enterotoxin, 10 μg/ml insulin, 0.5 μg/ml hydrocortisol, 20 ng/ml epidermal growth factor, and 5% horse serum) and supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin [6-9]. All the cultures were maintained in 5% CO_2 at 37°C. Stock aqueous solutions of NNK (Chemsyn, Lenexa, KS), B[a]P (Aldrich, Milwaukee, WI), and chloromethyl-dichlorodihydro-fluorescein-diacetate (CM-H_2DCF-DA) (Invitrogen, Carlsbad, CA) were prepared in DMSO and diluted with culture medium for assays. GTC (Polyphenon-60, a mixture of polyphenolic compounds containing 60% total catechins, Sigma, St. Louis, MO) was prepared in distilled water and diluted with medium.

Induction and Suppression of Cell Carcinogenesis

Twenty-four hours after each subculturing, MCF10A cells were treated with combined NNK and B[a]P each at 100 pmol/L in the absence and presence of GTC for 48 h as one cycle of exposure for 10 cycles; cultures were subcultured every 3 d.

Cell Viability Assay

A Methyl Thiazolyl Tetrazolium (MTT) assay kit (ATCC) was used to measure cell growth and viability in cultures. Five x 10^3 cells were seeded into each well of 96-well culture plates. After treatments, cultures were incubated with MTT reagent for 4 h, followed by incubation with detergent reagent for 24 h. Reduced MTT reagent in cultures was quantified with an ELISA reader (Bio-Tek, Winooski, VT).

Cell Proliferation Assay

Five x 10^3 cells were seeded into each well of 96-well culture plates. After treatments, using the 5-bromo-2-deoxyuridine (BrdU) cell proliferation ELISA kit (Roche, Indianapolis, IN), cultures were labeled with BrdU for 12 h, fixed, incubated with peroxidase-conjugated BrdU-specific antibodies, and stained with the peroxidase substrate. Quantification of BrdU-labeled cells was determined with an ELISA reader (Bio-Tek).

Apoptosis Assay

An annexin-V-fluorescein isothiocyanate (FITC) apoptosis detection kit with propidium iodide (BD Biosciences, San Jose, CA) was used to detect apoptotic cell death by flow cytometry, as described previously [33]. Flow cytometric analysis was performed on the Coulter EPICS Elite Cytometer (Hialeah, FL) at the excitation and emission wavelengths of 488 and 550 nm, respectively, for FITC measurements, and at 488 and 645 nm for propidium iodide measurements. Percentage of cells undergoing apoptosis was determined using Multicycle software (Phoenix Flow System, San Diego, CA).

Reduced Dependence on Growth Factors Assay

The low-mitogen (LM) medium contained total serum and mitogenic additives reduced to 2% of the concentration formulated in CM medium as described above [7-9]. Three $\times 10^3$ cells were seeded in LM medium; growing colonies that reached 0.5 mm diameter in 10 d were counted.

Anchorage-independent Cell Growth Assay

The base layer consisted of 2% low-melting agarose (Sigma) in CM medium. Then, soft agar consisting of 0.4% low-melting agarose in a mixture (1:1) of CM medium with 3-d conditioned medium prepared from MCF10A cultures was mixed with 5×10^3 cells and plated on top of the base layer in 60-mm diameter culture dishes [7-9]; growing colonies that reached 0.1 mm diameter in 20 d were counted.

Acinar-Conformational Disruption Assay

Placed as a Matrigel base of reconstituted basement membrane in each well of 24-well culture plates was 400 mL of growth factor-reduced Matrigel Matrix (BD Biosciences) [7, 8, 34]. Two × 10^3 cells were mixed with CM medium containing 4% Matrigel and plated on top of the base layer of Matrigel. Cultures were maintained in 5% CO_2 at37°C and were replaced with fresh CM medium containing 2% Matrigel every 3 d for 14 d. Spheroids in Matrigel were collected and overlaid with 5% agarose; then blocks of agarose-packed Matrigel spheroids were fixed in neutral-buffered formalin and embedded in paraffin for histological examination of 5-mm H&E-stained sections.

Cell Motility Assay

Cells were grown to confluence in CM medium, rinsed with PBS, and maintained in DMEM/Ham's F12 media supplemented with 2% serum for 15 h [35]. The monolayer was scratched with a 23-gauge needle (BD Biosciences) to generate wounded areas and rinsed with CM medium to remove floating cells. Cultures were maintained in CM medium, and the wounded areas were examined 6 h and 24 h after scratches. Wound healing area was calculated by using Total Lab TL100 software (Total Lab, Newcastle, NE).

Intracellular ROS Measurement

Cultures were labeled with 5 μmol/L CM-H_2DCF-DA for 1 h [36]. Cells were trypsinized from cultures and resuspended in PBS for analysis of ROS by flow cytometry, using a 15 mW, air-cooled argon laser to produce 488 nm light. DCF fluorescence emission was collected with a 529 nm band pass filter. The mean fluorescence intensity of 2×10^4 cells was quantified using Multicycle software (Phoenix Flow System).

Western Immunoblotting

Cell lysates were prepared in buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM sodium pyrophosphate, 10% glycerol, 0.1% Na_3VO_4, 50 mM NaF, pH 7.4) [8,9]. Protein concentration in cell lysates was measured using the BCA assay (Pierce, Rockford, IL). Equal amounts of cellular proteins were resolved by electrophoresis in 10 or 12% SDS-polyacrylamide gels for Western immunoblotting with antibodies specific to phosphorylated Mek1/2 (p-Mek1/2), Mek1/2, p-Erk1/2, p-H2AX, H2AX (Cell Signaling, Beverly, MA), Erk1/2, and β-Actin (Santa Cruz, Santa Cruz, CA). Antigen-antibody complexes on filters were detected by the Supersignal chemiluminescence kit (Pierce).

Gene Expression Study with Microarrays

MCF10A cells were treated with combined NNK and B[a]P each at 100 pmol/L in the absence or presence of 40 μg/mL of GTC for 24 h. Total RNA was isolated from cultures using the Absolutely RNA kit (Stratagene, La Jolla, CA). RNA quality and integrity were determined

using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). High-quality RNA, with an RNA Integrity Number of >7.0 and an A260/280 absorbance ratio of >1.8, was used for studies. Detection of gene expression was completed through a purchased service using the Human OneArray, which contains 29,187 human genome probes and 1088 experimental control probes formed as 60-mer sense-strand DNA elements (PhalanxBio, Palo Alto, CA). All experiments were performed in duplicate; RNA was prepared from two independent cultures for each experiment. The data was analyzed using Array Studio Online (OmicSoft, Morrisville, NC). The relative gene expression levels in carcinogen- and GTC-treated cultures were normalized with levels in their counterpart, parental MCF10A cells and then were reciprocally compared to detect differentially expressed genes in GTC- and carcinogen (only)-treated cultures versus carcinogen-treated cultures.

Reverse Transcription PCR

One μg of total RNA isolated from cultures using the Absolutely RNA kit (Stratagene) was reverse transcribed to cDNA using a Versoc DNA Kit (Thermo Scientific, Waltham, MA). The resulting cDNAs were subjected to PCR for COX17 (forward: 5'-TCTAATTGAGGCCCACAAGG-3'; reverse: 5'-ATTCACACAGCAGACCACCA-3'), TNFRSF8 (forward: 5'-AAACCGCTCAGATGTTTTGG-3'; reverse: 5'-TGATGCAGAGACACCCACTC-3'), S100P (forward: 5'-TCCTGCAGAGTGGAAAAGAC-3'; reverse: 5'-TAGGGGAATAATTGCCAACA-3'), ATM (forward: 5'-ACTGCCAAGGACAAATGAGG-3'; reverse: 5'-TGAGCAACTGAGTGGCAAAC-3'), and β-Actin (forward: 5'-GGACTTCGAGCAAGAGATGG-3'; reverse: 5'-

AGCACTGTGTTGGCGTACAG-3'). PCR was carried out as follows: 1 cycle at 95°C for 2 min, 45 cycles at 95°C for 30 s and 58°C for 45 s, and the final extension of 1 cycle at 72°C for 30 s. PCR products were electrophoresed on 2% agarose gel and visualized after ethidium bromide staining.

Statistical Analysis

To statistically verify the suppression of NNK- and B[a]P-induced carcinogenesis by GTC, one-way analysis of variance (ANOVA) test was used to establish significant difference among the various treatment groups at $P < 0.01$, followed by Duncan's Multiple Range Test. Statistical significance of all the other studies was analyzed by Student *t* test ($P \leq 0.05$) and α levels were adjusted by the Simes method [37].

Results and Discussion

Determination of GTC Cytotoxicity

Considering the side-effects resulting from long-term use of anticancer agents in intervention of cellular carcinogenesis, use of non-cytotoxic levels of dietary components has to be adopted into strategies for cancer prevention. To determine the cytotoxicity of GTC to non-cancerous breast epithelial cells, we investigated the biological effects of GTC at various concentrations on viability, proliferation, and cell death of MCF10A cells. We detected cytotoxic

activity of GTC at 100 μg/mL, but not at 0.5, 2.5, 10, and 40 μg/mL, in reducing cell viability (**Figure 2.1A**), inhibiting cell proliferation (**2.1B**), or inducing apoptotic cell death (**2.1C**). Accordingly, GTC at 0.5, 2.5, 10, and 40 μg/mL was non-cytotoxic to MCF10A cells.

GTC Suppression of NNK- and B[a]P-induced Carcinogenesis

In our chronic carcinogenesis model, cumulative exposures of MCF10A cells to pico-molar NNK and B[a]P result in progressive acquisition of various cancer-associated properties [7-9]. Using cancer-associated properties as target endpoints, we investigated the activity of GTC in suppression of NNK- and B[a]P-induced cellular carcinogenesis. A lack of growth factors causes normal cells to become growth-arrested in the cell cycle and to commit apoptosis; however, aberrantly-increased cell survivability acquired to reduce dependence on growth factors can lead cells to tumorigenic transformation [38-40]. Cell adhesion to extracellular matrixes is important for cell survival in a multi-cell environment; aberrantly-increased cell survivability acquired to promote anchorage-independent growth can render cells into tumorigenic transformation [41,42]. Cancerous cells acquire an increased mobility compared with their normal counterpart cells [43]. MCF10A cells were exposed to NNK combined with B[a]P in the presence of GTC at 0, 0.5, 2.5, 10, and 40 μg/mL for 10 cycles, resulting in NB, NB/G-0.5, NB/G-2.5, NB/G-10, and NB/G-40 cell lines, respectively. We detected that GTC at 2.5, 10, and 40 μg/mL significantly suppressed NNK- and B[a]P-induced acquisition of the cancer-associated property of reduced dependence on growth factors to approximately 20, 40, and 75%, respectively (**Figure 2.2A-1**), as well as anchorage-independent growth to approximately 20, 50, and 80%, respectively (**2.2B-1**). Not only the numbers but also sizes of

cell colonies were suppressed by GTC (**Figure 2.2A-2** and **2.2B-2**). Using the scratch/wound assay [35], we detected that NB cells acquired high mobility to heal the wounded area in 24 h; in contrast, parental MCF10A cells did not heal the wounded area; and NB/G-0.5, NB/G-2.5, NB/G-10, and NB/G-40 cells showed various abilities to heal the wounded areas (**Figure 2.2C-1**). Thus, co-exposure to GTC significantly reduced NNK- and B[a]P-induced acquisition of the cancer-associated property of increased cell mobility in a dose-dependent manner (**Figure 2.2C-2**).

In addition, both acinar structures with a hollow lumen and apicobasally polarized cells are important characteristics found in glandular epithelia in vivo; the disruption of an intact glandular structure is a hallmark of epithelial cancer even at precancerous stages [44,45]. As shown in our previous studies [7-9], parental MCF10A cells mainly formed regular, round spheroids on Matrigel cultures, and NNK-and B[a]P-exposed cells formed both regular and irregular spheroids. Counting the regular and irregular spheroids in these cultures verified the ability of GTC at 2.5, 10, and 40 μg/mL to significantly suppress NNK- and B[a]P-induced development of irregular spheroids to approximately 10, 45, and 65%, respectively (**Figure 2.2D-1**). Histological examination revealed a hollow lumen and apicobasal polarization in regular spheroids of MCF10A cells and GTC-protected, carcinogen-exposed cells (NB/G-40) as well as the loss of apicobasal polarity and filling of the luminal space in irregular spheroids of NNK- and B[a]P-exposed cells (NB) and NB/G-40 cells on Matrigel (**Figure 2.2D-2**). These results indicated that GTC was able to protect MCF10A cells from acquiring the cancer-associated property of acinar-conformational disruption induced by NNK and B[a]P in a dose-dependent manner. Analysis of all these results together indicated that GTC at 2.5, 10, and 40 μg/mL was effective to significantly suppress NNK- and B[a]P-induced cellular carcinogenesis

quantitatively and qualitatively. Clinical and animal studies showed an achievable plasma level of GTC at ≈4 μg/mL [46-49]. Therefore, GTC at 2.5 μg/mL can be bio-availably achieved for dietary intervention and chemoprevention of breast cell carcinogenesis associated with long-term exposure to environmental carcinogens.

MCF10A cells repeatedly treated with combined NNK and B[a]P each at 100 pmol/L for 10 cycles acquired various cancer-associated properties but did not acquire tumorigenicity to develop any detectable xenograft tumors in immunodeficient nude mice (data not shown). It has been shown that NNK is able to induce mammary tumors in rats [13], and it is recognized that exposure to B[a]P contributes to sporadic breast cancer development [17-20]. Although NNK and B[a]P are recognized as precancerous breast carcinogens in our model, whether additional exposures to NNK and B[a]P may induce cellular tumorigenicity remains to be determined. Thus, our model is able to detect precancerous carcinogenesis of breast cells and identify preventive agents to intervene in precancerous breast cell carcinogenesis for early prevention of breast cancer associated with long-term exposure to low doses of environmental and tobacco carcinogens.

GTC Suppression of Carcinogen-induced ROS, Cell Proliferation, the ERK Pathway, and H2AX Phosphorylation

Short-term exposure of MCF10A cells to the B[a]P metabolites B[a]P-quinones at 10 μmol/L induces ROS [50]. Short-term exposure of normal human bronchial epithelial cells to NNK at 1-5 μmol/L induces cell proliferation [51]. It has been postulated that ROS elevation and increased proliferation may promote cell susceptibility to DNA damage induced by carcinogens,

contributing to cellular carcinogenesis [52,53]. However, it is not clear whether MCF10A cell carcinogenesis induced by cumulative exposures to NNK and B[a]P at 100 pmol/L is contributed by ROS elevation, proliferation, and DNA damage during each exposure. To address this question and to pursue the mechanisms for GTC in counteracting the activity of NNK and B[a]P in initiation of cellular carcinogenesis, we studied the activity of GTC in modulation of NNK- and B[a]P-induced ROS elevation, cell proliferation, proliferation-associated signaling pathways, and DNA damage. As shown in **Figure 2.3**, treatment with NNK and B[a]P for 24 h induced ROS elevation (**A**), cell proliferation (**B**), activation of the ERK pathway (indexed by phosphorylation of Mek1/2 and Erk1/2) [54] (**C**), and DNA damage (indexed by phosphorylation of H2AX on serine-139) [55] (C). Co-treatment with GTC at 2.5, 10, and 40 μg/mL resulted in a dose-dependent reduction of these carcinogen-induced biological and biochemical outcomes. Accordingly, suppression of carcinogen-induced ROS elevation, cell proliferation, ERK activation, and DNA damage may account for the mechanism of GTC in intervention of cellular carcinogenesis. However, the targets involved in ROS elevation, cell proliferation, ERK activation, and DNA damage for GTC suppression of NNK- and B[a]P-initiated carcinogenesis are to be identified.

GTC Intervention of Carcinogen-induced Gene Expression

To further our investigation of GTC activity in intervention of NNK- and B[a]P-initiated carcinogenesis, we used cDNA microarrays to detect differentially regulated genes that were changed in carcinogen-treated cells but whose changes were suppressed by GTC. Initially, we identified genes whose expressions were changed in carcinogen-treated cells compared to their

counterpart expression levels in untreated cells. After normalization, more than 11,000 genes were detectably expressed in these cells. Filtering with the *t* test ($P < 0.05$) revealed 479 differentially expressed genes in carcinogen-treated cells, more than 2-fold over counterpart expression in untreated, counterpart cells. Subsequently, we identified genes whose expressions were associated with ROS elevation, cell proliferation, the ERK pathway activation, and DNA damage, but were not induced in GTC- and carcinogen-treated cells. As listed in **Table 2.1**, we detected that three genes (BAX, COX17, and MRPL41) associated with ROS elevation, four genes (B4GALT1, BARHL1, BOLA3, and MT1E) associated with cell proliferation, two genes (S100P and SPRR1B) associated with ERK pathway activation, and two genes (ATM and PER1) associated with DNA damage were up-regulated in carcinogen-treated cells but their up-regulations were suppressed in GTC- and carcinogen-treated cells; in addition, one gene (TNFRSF8) associated with negative regulation of cell proliferation was down-regulated in carcinogen-treated cells but not down-regulated in GTC- and carcinogen-treated cells. To further validate microarray results with an independent method, we arbitrarily chose a gene from each category for reverse transcription PCR semi-quantification to measure relative gene expression levels in cells treated with carcinogens and/or GTC. As shown in **Figure 2.4**, gene expression of COX17, S100P and ATM, which were associated with ROS elevation, ERK pathway activation, and DNA damage, respectively, were increased by NNK and B[a]P treatment; but NNK- and B[a]P-increased expression of these genes was significantly reduced by co-treatment with GTC. In contrast, TNFRSF8 gene expression associated with cell proliferation was reduced in cells treated with NNK and B[a]P and up-regulated in cultures treated with GTC alone or GTC and carcinogens. The PCR results were consistent with microarray data. Accordingly, expression of these genes may be considered as a molecular target endpoint for GTC intervention of NNK- and

B[a]P-initiated cellular carcinogenesis. However, their roles in carcinogen-induced ROS elevation, cell proliferation, ERK activation, and DNA damage and GTC-induced suppression of carcinogenesis are to be identified.

Our model presents unique features of chronic induction of breast cell carcinogenesis by cumulative exposures to carcinogens, high sensitivity to detect low levels of carcinogens, and measurable target endpoints. Applying this cellular model as a target will accelerate the identification of dietary components for the formulation of combined supplements that can effectively reduce the health risk of human cancers from long-term exposure to carcinogens present in environmental pollution.

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APPENDIX

Table 2.1

Table 2.1. Genes Up- and Down-regulated by NNK and B[a]P and Protected by GTC

Gene name	Function	Changes induced by NNK & B[a]P
Expression associated with ROS elevation		
BAX (BCL2-associated X protein)	Regulated by ROS via increasing promoter activity [56]	↑
COX17 (Cytochrome c oxidase assembly homolog-17)	Regulate ROS elevation by increasing activity of cytochrome c oxidase [57]	↑
MRPL41 (Mitochondrial ribosomal protein L41)	Induced by ROS to help stabilize p53 [58]	↑
Expression associated with cell proliferation		
B4GALT1 (BetaGlcNAc beta 1,4-galactosyltransferase-1)	Increase proliferation by enhancing estrogen expression [59]	↑

Table 2.1. Genes Up- and Down-regulated by NNK and B[a]P and Protected by GTC

Gene name	Function	Changes induced by NNK & B[a]P
BOLA3 (BolA homolog-3)	Increase proliferation by regulating cell cycle [61]	↑
MT1E (Metallothionein 1E)	Induce proliferation and migration of bladder cancer cells [62]	↑
TNFRSF8 (Tumor necrosis factor receptor superfamily-8)	Decrease proliferation and increase apoptosis [63]	↓
Expression associated with ERK pathway activation		
S100P (S100 calcium binding protein P)	Regulate Erk phosphorylation in colon cancer cells [64]	↑
SPRR1B (Small proline-rich protein 1B)	Regulated by ERK pathway in epithelial cells [65]	↑

Table 2.1. Genes Up- and Down-regulated by NNK and B[a]P and Protected by GTC

Gene name	Function	Changes induced by NNK & B[a]P
Expression associated with DNA damage		
ATM (Ataxia telangiectasia mutated)	Induced by double strand DNA damage [55]	↑
PER1 (Period homolog-1)	Induce DNA damage in human cancer cells [66]	↑

↑ up-regulation; ↓ down-regulation

Figure 2.1. Determination of GTC cytotoxicity. MCF10A cells were treated with 0, 0.5, 2.5, 10, 40, and 100 μg/mL of GTC for 48 h. (A) Quantification of cell viability was determined with an MTT assay kit, and relative cell viability was normalized by the value determined in untreated counterpart cells, set as 100%. (B) Cell proliferation was determined; relative cell growth rate was normalized by the value of BrdU detected in untreated cells, set as 100%. (C) Apoptotic cell population (%) was measured by flow cytometry with an Annexin-V-FITC Apoptosis Detection Kit. *Columns*, mean of triplicates; *bars*, SD. The Student *t* test was used to compare the control with each of the treatments to analyze statistical significance and *P* values adjusted for multiple comparisons using the Simes method, indicated by ** $P < 0.01$. All results are representative of three independent experiments.

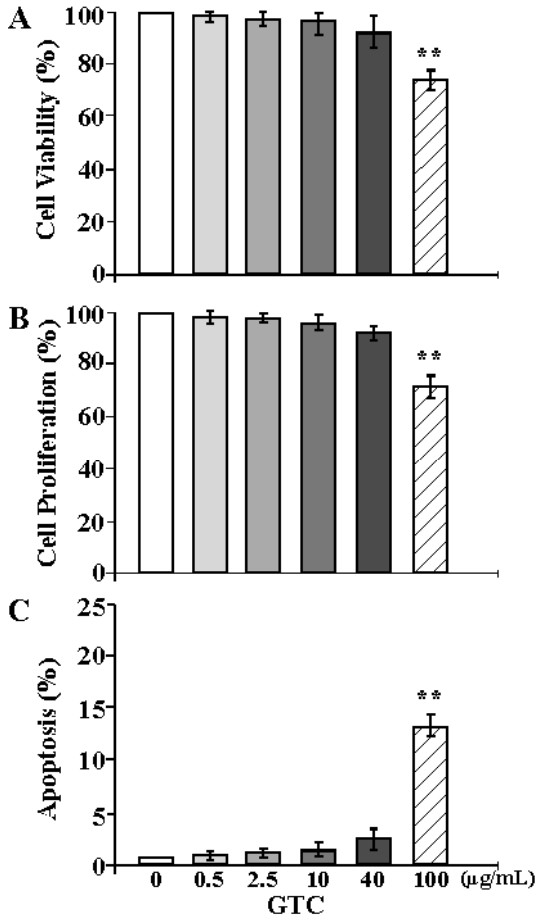
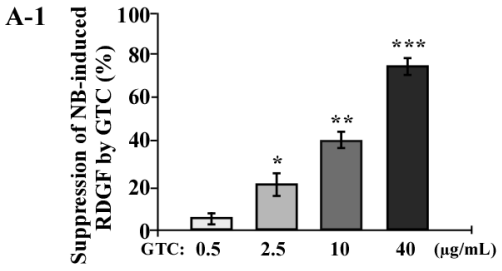


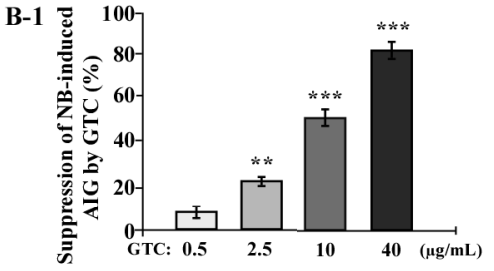
Figure 2.1.

Determination of GTC cytotoxicity.

Figure 2.2. GTC suppression of cellular carcinogenesis. MCF10A cells were exposed to NNK combined with B[a]P each at 100 pmol/L in the presence of GTC at 0, 0.5, 2.5, 10, and 40μg/mL for 10 cycles, resulting in NB, NB/G-0.5, NB/G-2.5, NB/G-10, and NB/G-40 cell lines, respectively. (A-1 and A-2) To detect suppression effectivity of GTC on cellular acquisition of reduced dependence on growth factors (RDGF), cells were then seeded and maintained in LM medium for 10 d. Cell colonies (≥ 0.5 mm diameter) were stained and counted. (B-1 and B-2) To detect suppression effectivity of GTC on cellular acquisition of anchorage-independent growth (AIG), cells were seeded in soft-agar for 20 d. Colonies (≥ 0.1 mm diameter) were counted. The percentage of suppression effectivity of GTC at various concentrations on NB-induced RDGF (A-1) and AIG (B-1) were calculated by: {1 − [(# of NB/G-induced cell colonies) − (# of MCF10A cell colonies)] ÷ [(# of NB-induced cell colonies) − (# of MCF10A cell colonies)]} x 100. *Columns*, mean of triplicates; *bars*, SD. The Student *t* test was used to compare the control with each treatment to analyze statistical significance and *P* values adjusted for multiple comparisons using the Simes method, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (A-2 and B-2) colony numbers, average colony size, and range of colony size were determined in A-1 and B-1, respectively. Mean colony numbers in each treatment were analyzed by one-way ANOVA at $P < 0.01$ to indicate significant difference in number of colonies in various treatments. To further determine significant difference between individual treatments, a pairwise analysis of variables was performed using the Duncan multiple range test. Significant differences at $P < 0.01$ between treatments are labeled with different superscript letters (a, b, c, d, and e); treatments labeled with the same superscript letters indicate no significant difference. (C-1 and C-2) To detect suppression effectivity of GTC on cellular acquisition of increased cell mobility, cells were seeded in CM medium and grown to confluence (**a**). A linear area of cell layer was

removed from each culture with a 23-gauge needle to produce wounded cultures, and the wounded areas were examined (×100 magnification) 6 h (**b**) and 24 h (**c**) after wounding. Arrows indicate width of wounded areas. Results are representative of three independent experiments. (C-2) To calculate the wound healing area, the area not healed by the cells was subtracted from total area of initial wound to calculate the wound healing area at time intervals of 6 h (white columns) and 24 h (hatched columns). (D-1 and D-2) To detect suppression effectivity of GTC on cellular acquisition of acinar-conformation disruption (ACD), cells were seeded on Matrigel for 12 d. Regular and irregular spheroids in each Matrigel culture were counted; and then the percentage of irregular spheroids in each culture was calculated. The value of the suppression effectivity of GTC on carcinogen-induced formation of irregular spheroids was calculated by: {[(% of NB-induced irregular spheroid population) − (% of NB/G-induced irregular spheroid population)] ÷ [(% of NB-induced irregular spheroid population) − (% of irregular spheroid population in MCF10A cultures)]} x 100 (%). *Columns*, mean of triplicates; *bars*, SD. The Student *t* test was used to compare the control with each treatment to analyze statistical significance and *P* values were adjusted for multiple comparisons using the Simes method, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All results are representative of three independent experiments. (D-2) Histological examination revealed acinar features of typical regular spheroids of MCF10A, regular spheroids of NB/G-40, and irregular spheroids of NB and NB/G-40 cells on Matrigel. Bars indicate50 μm.





A-2

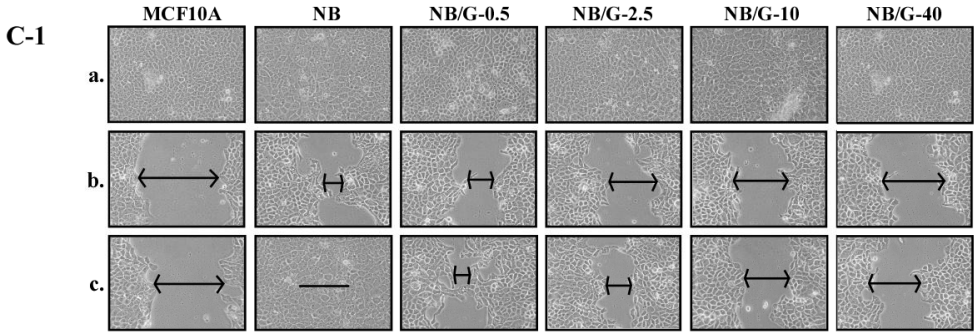
	Colony Number	Colony Size	
		Average, mm	Range, mm
MCF10A	50 ± 4^{e}	0.62	0.5-0.8
NB	142 ± 6^{a}	1.34	0.8-1.7
NB/G-0.5	138 ± 3^{a}	1.31	0.8-1.6
NB/G-2.5	123 ± 5^{b}	1.25	0.8-1.6
NB/G-10	102 ± 6^{c}	0.83	0.6-1.2
NB/G-40	75 ± 7^{d}	0.72	0.5-1.0

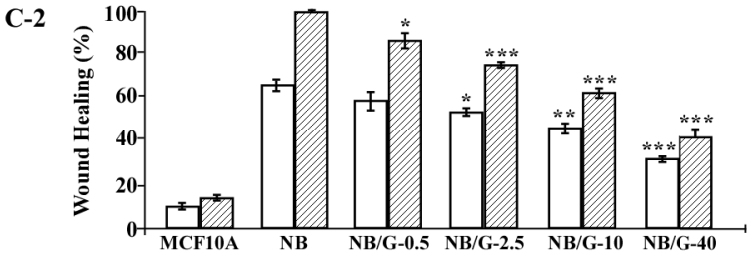
(Mean ± SD, n=5, P<0.001 one-way ANOVA with pairwise comparison with Duncan's Multiple Range Test. Different superscript letters indicate significant difference.)

B-2

	Colony Number	Colony Size	
		Average, mm	Range, mm
MCF10A	5 ± 2^{e}	0.22	0.1-0.3
NB	94 ± 5^{a}	0.58	0.2-0.9
NB/G-0.5	91 ± 2^{a}	0.57	0.2-0.9
NB/G-2.5	75 ± 3^{b}	0.54	0.2-0.8
NB/G-10	42 ± 3^{c}	0.43	0.1-0.6
NB/G-40	25 ± 4^{d}	0.35	0.1-0.4

(Mean ± SD, n=5, P<0.001 one-way ANOVA with pairwise comparison with Duncan's Multiple Range Test. Different superscript letters indicate significant difference.)





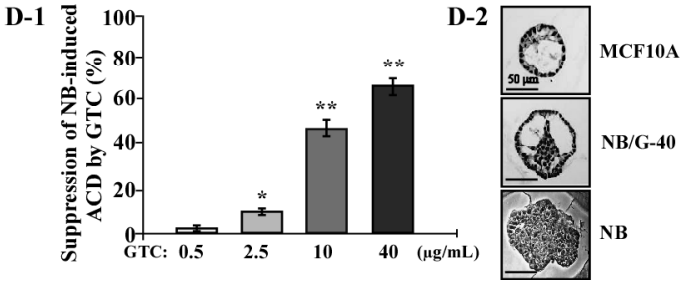


Figure 2.2.

GTC suppression of cellular carcinogenesis

Figure 2.3. GTC suppression of carcinogen-induced ROS elevation, cell proliferation, the ERK pathway, and phosphorylation of H2AX. MCF10A cells were treated with combined NNK and B[a]P (NB) each at 100 pmol/L in the presence of 0, 0.5, 2.5, 10, and 40 μg/mL of GTC for 24 h. (A) ROS levels were measured with CM-H_2DCF-DA labeling; relative level of ROS, as fold induction (X, arbitrary unit), was normalized by the level determined in untreated counterpart cells, set as 1. (B) Cell proliferation was determined; relative cell growth rate was normalized by the value of BrdU detected in untreated counterpart cells, set as 1. *Columns*, mean of triplicates; *bars*, SD. The Student *t* test was used to compare the control with each treatment to analyze statistical significance and *P* values adjusted for multiple comparisons using the Simes method, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (C) Cell lysates were prepared and analyzed by Western immunoblotting to detect levels of phosphorylated Mek1/2 (p-Mek1/2), Mek1/2, p-Erk1/2, Erk1/2, p-H2AX, H2AX, with β-Actin as a control. Levels of p-Mek1/2, Mek1/2, p-Erk1/2, Erk1/2, p-H2AX, H2AX, and β-Actin were quantified by densitometry. Levels of specific phosphorylation of Mek1/2 (p-Mek1/2/Mek1/2), Erk1/2 (p-Erk1/2/Erk1/2), and H2AX (p-H2AX/H2AX) were calculated by normalizing levels of p-Mek1/2, p-Erk1/2, and p-H2AX with levels of Mek1/2, Erk1/2, and H2AX, respectively, and then further normalizing with β-Actin level and the level set in untreated cells (lane1) as 1 (X, arbitrary unit). All results are representative of three independent experiments.

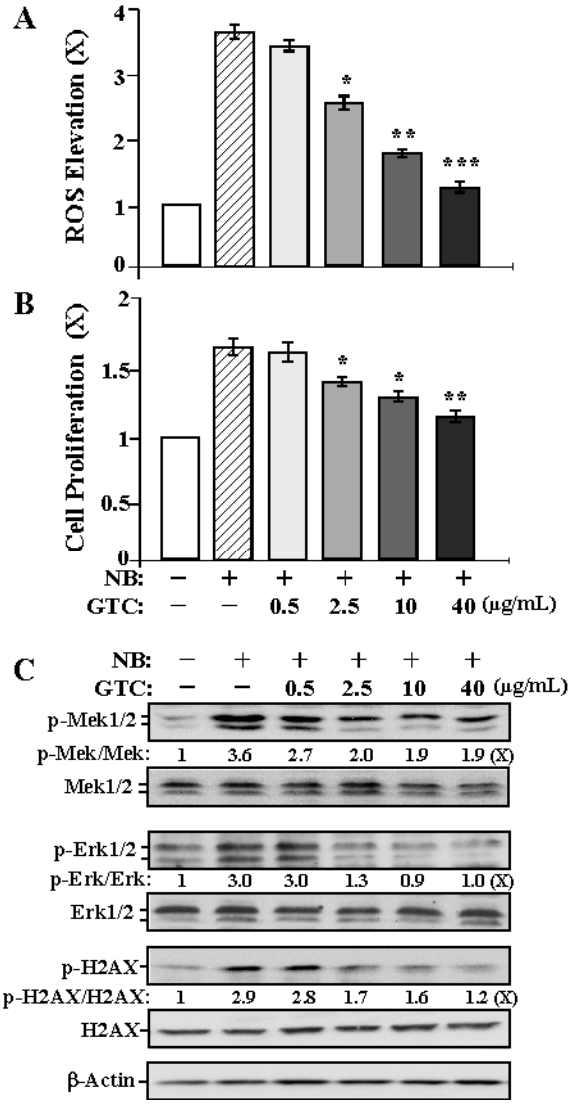


Figure 2.3.

GTC suppression of carcinogen-induced ROS elevation, cell proliferation, the ERK pathway, and phosphorylation of H2AX

Figure 2.4. GTC intervention of carcinogen-induced gene expression. MCF10A cells were treated with combined NNK and B[a]P (NB) each at 100 pmol/L in the presence of 40 μg/mL of GTC for 24 h. Total RNAs were isolated and analyzed by reverse transcription PCR with specific primers to determine relative gene expression levels of COX17, S100P, ATM, and TNFRS8, with β-Actin as a control. Total gene expression levels of COX17, S100P, ATM, TNFRS8, and β-Actin were quantified by densitometry. Relative gene expression levels were calculated by normalizing the levels of COX17, S100P, ATM, and TNFRS8 gene expression with β-Actin level and the level set in untreated cells (lane1) as 1 (X, arbitrary unit). All results are representative of three independent experiments.

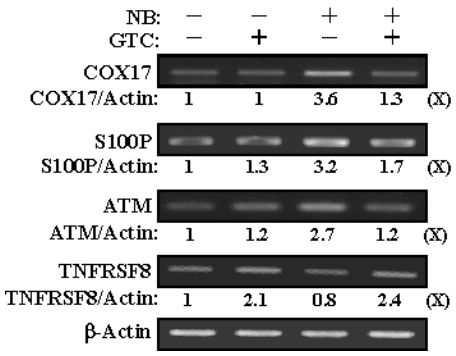


Figure 2.4.

GTC intervention of carcinogen-induced gene expression

PART –III

Green tea catechin intervention of reactive oxygen species-mediated erk pathway activation and chronically-induced breast cell carcinogenesis

Research described in this chapter is submitted for publication in Carcinogenesis by Kusum Rathore, Shambhunath Choudhary, Agricola Odoi and Hwa-Chain Robert Wang

Kusum Rathore, Shambhunath Choudhary, Agricola Odoi and Hwa-Chain Robert Wang.
Green tea catechin intervention of reactive oxygen species-mediated ERK pathway activation and chronically-induced breast cell carcinogenesis.
(Carcinogenesis 2011; doi: 10.1093/carcin/bgr244)

In this paper “our” and “we” refers to me and co-authors. My contribution in the paper includes (1) Selection of the topic (2) Compiling and interpretation of the literature (3) Designing experiments (4) understanding the literature and interpretation of the results (5) providing comprehensive structure to the paper (6) Preparation of the graphs and figures (7) Writing and editing

Abstract

Long-term exposure to low doses of environmental carcinogens contributes to sporadic human breast cancers. Epidemiologic and experimental studies indicate that green tea catechins (GTCs) may intervene with breast cancer development. We have been developing a chronically-induced breast cell carcinogenesis model wherein we repeatedly expose non-cancerous, human breast epithelial MCF10A cells to bio-achievable pico-molar concentrations of environmental carcinogens, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a]P), to progressively induce cellular acquisition of cancer-associated properties, as measurable endpoints. The model is then used as a target to identify non-cytotoxic preventive agents effective in suppression of cellular carcinogenesis. Here, we demonstrate, for the first time, a two-step strategy that initially used endpoints that were transiently-induced by short-term exposure to NNK and B[a]P as targets to detect GTCs capable of blocking the acquisition of cancer-associated properties, and subsequently used endpoints constantly-induced by long-term exposure to carcinogens as targets to verify GTCs capable of suppressing carcinogenesis. We detected that short-term exposure to NNK and B[a]P resulted in elevation of reactive oxygen species (ROS), leading to Raf-independent ERK pathway activation and subsequent induction of cell proliferation and DNA damage. These GTCs, at non-cytotoxic levels, were able to suppress chronically-induced cellular carcinogenesis by blocking carcinogen-induced, ROS elevation, ERK activation, cell proliferation, and DNA damage in each exposure cycle. Our model may help accelerate the identification of preventive agents to intervene in carcinogenesis induced by long-term exposure to environmental carcinogens, thereby safely and effectively reducing the health risk of sporadic breast cancer.

Introduction

More than 70% of sporadic breast cancers are attributable to long-term exposure to environmental factors, such as chemical carcinogens, etc; this multi-year, multi-step, and multi-path disease process involves cumulative genetic and epigenetic alterations to induce progressive carcinogenesis of breast cells from non-cancerous to precancerous and cancerous stages (1-4). Over 200 chemical mammary carcinogens have been experimentally detected to acutely induce cancerous cells in cultures and tumors in animals at high doses of micro- to milli-molar concentrations (1,3,5). A high-dose approach may serve as a proper way to study occupational exposure; however, considering that chronic exposure of human tissues to low doses of carcinogens is responsible for most human cancers, a chronic, low-dose approach might be a more proper way to study the environmental exposure most often responsible for human breast cancer development. A new approach is needed to reveal environmental, mammary carcinogens, at low and bio-achievable levels, capable of inducing human breast cell carcinogenesis.

We have been developing a model to mimic breast cell carcinogenesis occurring with accumulated exposures to low doses of environmental carcinogens (6-9). We used the environmental carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a]P) at pico-molar concentrations, like those detected in patients (10-13), to repeatedly treat immortalized, non-cancerous, human breast epithelial MCF10A cells in culture to progressively induce acquisition of cancer-associated properties (6-9). NNK is considered one of the most potent lung carcinogens in tobacco products (14); although gastric administration of NNK into rats results in DNA-adduct formation in the mammary gland and development of mammary tumors (14-17), NNK is not currently recognized as a breast carcinogen. B[a]P, a family member of polycyclic aromatic hydrocarbons, is considered an environmental, dietary,

and tobacco carcinogen, its metabolites forming strong DNA adducts and causing DNA lesions, and it is recognized as a mammary carcinogen in rodents (3,4,12,13,18-21). Studies using human cell lines for genotoxicity tests and studies of adduct formation reveal genotoxic activity of NNK and B[a]P at concentrations as low as 25 mmol/L and 25 μmol/L, respectively (22). Our cellular model reveals the ability of NNK and B[a]P, at a bio-achievable level of 100 pmol/L, to chronically and progressively induce carcinogenesis of MCF10A cells (6-9). Hence, our model system takes a new, sensitive approach of validating low doses of environmental, mammary carcinogens in chronic induction of human breast cell carcinogenesis.

It has been shown that a short-term exposure of MCF10A cells to the B[a]P metabolites B[a]P-quinones at 10 μmol/L for 10 min induces reactive oxygen species (ROS) elevation (23), and exposure of normal human bronchial epithelial cells to NNK at 1 to 5 μmol/L for 24 h induces cell proliferation (24). It has been postulated that ROS elevation and cell proliferation increase cell susceptibility to DNA damage induced by carcinogens, contributing to cellular carcinogenesis (25,26). The oxidative DNA damage caused by ROS includes strand breaks and nucleotide modifications, resulting in mutations and contributing to cellular transformation (19). Activation of the ERK pathway also contributes to cell proliferation and phosphorylation of histone H2AX (27), the latter of which (on serine 139) is widely used as an indicator for DNA damage (28). In addition, B[a]P, at high doses ranging from 0.02 to 1 μmol/L, has been shown to induce cell proliferation and DNA damage in breast adenocarcinoma MCF7 cells (29,30). However, it is not clear whether pico-molar levels of NNK and B[a]P are able to induce ROS elevation and cell proliferation in breast cells with short-term exposure, contributing to induction of carcinogenesis associated with long-term exposure.

Epidemiologic and experimental studies have shown that various dietary polyphenolic compounds, which are widely found in vegetables, fruits, and tea, possess anticancer, antiproliferative, antioxidant, and apoptotic activities (1,31). The use of green tea to increase the body's antioxidant activity is becoming increasingly popular in the Western world (32). A typical brewed green tea contains 30 to 45% green tea catechins (GTCs), including epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG) (31). GTCs have been shown to be more effective antioxidants than vitamins C and E (33), and their order of effectiveness as radical scavengers is ECG > EGCG > EGC > EC (24). Animal studies show that GTCs are able to suppress rat mammary carcinogenesis induced by 7,12-dimethylbenz[a]anthracene and N-methyl-N-nitrosourea (34,35). Laboratory studies also have shown that GTCs possess inhibitory and apoptotic activity in the growth of human breast cancer cells in cultures (36,37). Epidemiological studies have examined the benefits of tea consumption for breast cancer prevention, and some evidence has indicated that green tea consumption may help prevent breast cancer recurrence in early stage cancers; however, the results are controversial (31,38,39). In addition, studies show that EGCG and EGC exhibit higher toxicity than ECG and EC in inducing cellular DNA damage (40,41). Therefore, additional studies are needed to clarify the effectivity of individual GTCs, at non-cytotoxic levels, used in protection of breast cells from carcinogenesis in order to safely and effectively reduce the health risk of sporadic breast cancer.

Previously, we used our model system to detect the ability of a dietary GTC extract containing 60% total catechins, at non-cytotoxic concentrations (< 40 μg/mL), to suppress chronically B[a]P-induced carcinogenesis of breast epithelial cells (8). In this study, we used our model system to pursue the mechanisms of NNK and B[a]P in inducing breast cell

carcinogenesis and identify targeted endpoints transiently or constantly induced by short-term and long-term exposure to both carcinogens, respectively. Then, we used NNK- and B[a]P-induced endpoints as targets to identify preventive agents capable of intervening in the cellular carcinogenesis. We identified the essential role of-ROS in modulating the ERK pathway leading to cell proliferation and chromosomal DNA damage in NNK- and B[a]P-induced breast cell carcinogenesis. We also revealed the preventive activity of individual EC, ECG, EGC, and EGCG, at non-cytotoxic levels, in suppression of NNK- and B[a]P-induced breast cell carcinogenesis.

Materials and methods

Cell cultures, reagents, and cellular carcinogenesis

Immortalized, non-cancerous human breast epithelial cell line MCF10A (American Type Culture Collection [ATCC], Rockville, MD) and derived cell lines were maintained in complete MCF10A culture medium (CM) (1:1 mixture of DMEM and HAM's F12, supplemented with 100 ng/mL cholera enterotoxin, 10 µg/mL insulin, 0.5 µg/mL hydrocortisol, 20 ng/mL epidermal growth factor, and 5% horse serum) (6-9). Human breast adenocarcinoma MCF7 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum. All cultures were maintained in medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin in 5% CO_2 at 37° C. Stock solutions of NNK (Chemsyn, Lenexa, KS), B[a]P (Aldrich, Milwaukee, WI), U0126 (Cell Signaling, Beverly, MA), and chloromethyl-dichlorodihydro-fluorescein-diacetate (CM-H_2DCF- DA) (Invitrogen, Carlsbad, CA) were

prepared in dimethyl sulfoxide and diluted with culture medium for assays. EC, ECG, EGC, EGCG (Sigma-Aldrich, St. Louis, MO), and *N*-acetyl-L-cysteine (NAC) (Alexis, San Diego, CA) were prepared in distilled water and diluted with culture media for assays.

Protocol for induction and suppression of cell carcinogenesis

To chronically induce cell carcinogenesis for intervention, 24 h after each subculturing, cultures were exposed to combined NNK and B[a]P each at 100 pmol/L in the absence and presence of individual EC, ECG, EGC, and EGCG for 48 h as one cycle of exposure for 5 to 20 cycles; cultures were subcultured every 3 days (3 days/cycle) (Figure 3.1A).

Assay for reduced dependence on growth factors

The low-mitogen (LM) medium contained reduced total serum and mitogenic additives to 2% of the concentration formulated in CM medium. Five × 10^3 cells were seeded in 100-mm culture dishes and maintained in LM medium. Growing cell colonies that reached 0.5 mm diameter in 10 days were stained with Coomassie brilliant blue and identified as cell clones acquiring the cancer-associated property of reduced dependence on growth factors.

Assay for anchorage-independent cell growth

The base layer consisted of 2% low-melting agarose (Sigma-Aldrich) in CM medium. Then, soft-agar consisting of 0.4% low-melting agarose in a mixture (1:1) of CM medium with 3 day-conditioned medium prepared from MCF10A cultures was mixed with 1 × 10^4 cells and

plated on top of the base layer in 60-mm diameter culture dishes. Growing colonies that reached 0.1 mm diameter by 20 days were identified as cell clones acquiring the cancer-associated property of anchorage-independent growth.

Cell mobility-healing assay

Cells were seeded in 6-well plates and grown to confluence in CM medium. Cells were rinsed with phosphate-buffered saline (PBS) and starved for 15 h in DMEM/Ham's F12 media containing 2% serum (42). The monolayer was then scratched with a 23-gauge needle (BD Sciences, Franklin Lakes, NJ) to generate wounded areas and rinsed with CM medium to remove floating cells. Cultures were maintained in CM medium, and the wounded areas were examined 6 h and 24 h after scratches to detect healing of wounded areas. Wound healing area was calculated by using Total Lab TL100 software (Total Lab, Newcastle, NE).

Cell viability assay

A methyl thiazolyl tetrazolium (MTT) assay kit (ATCC) was used to measure cell growth and viability in cultures. As described by the manufacturer, 3×10^4 cells were seeded into each well of 96-well culture plates for 24 h. After treatments, cells were incubated with MTT reagent for 4 h, followed by incubation with detergent reagent for 24 h. Quantification of reduced MTT reagent in cultures was determined with an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tek, Winooski, VT) at 570 nm.

Cell proliferation assay

Cell proliferation was determined using the 5-bromo-2-deoxyuridine (BrdU) cell proliferation ELISA kit (Roche, Indianapolis, IN). Five x 10^4 cells were seeded into each well of 96-well culture plates. After the indicated treatments, the cells were labeled with BrdU for 12 h, fixed, incubated with peroxidase-conjugated BrdU-specific antibodies, and stained with the peroxidase substrate. Quantification of BrdU-labeled cells was determined with an ELISA reader (Bio-Tek) at 370 nm.

Apoptotic cell death assay

An annexin-V-fluorescein isothiocyanate (FITC) apoptosis detection kit with propidium iodide (BD Sciences) was used to detect apoptotic cell death by flow cytometry (43). In brief, cells were collected after trypsinization and washed with PBS. Cells were then incubated with annexin-V-FITC and propidium iodide in a binding buffer (10 mmol/L Hepes-KOH, pH 7.4, 150 mmol/L NaCl, 1.8 mmol/L $CaCl_2$) for 20 min at ambient temperature in the dark. Flow cytometric analysis was performed on the Coulter EPICS Elite Cytometer (Hialeah, FL) at the excitation and emission wavelengths of 488 and 550 nm, respectively, for FITC measurements, and at 488 and 645 nm for propidium iodide measurements. The percentage of cells undergoing apoptotic death was determined using Multicycle software (Phoenix, San Diego, CA).

Measurement of ROS

To measure intracellular ROS levels, cells were incubated with 5 μmol/L CM-H_2DCF-DA for 1 h (44). Cells were rinsed with Ca^{++} and Mg^{++} free PBS, trypsinized from cultures, and resuspended in PBS for analysis of ROS by flow cytometry, as described above, using a 15 mW, air-cooled argon laser to produce 488 nm light. DCF fluorescence emission was collected with a 529-nm band pass filter. The mean fluorescence intensity of 2×10^4 cells was quantified using Multicycle software (Phoneix) .

DNA damage assay

DNA damage was detected with a comet assay. Cells were trypsinized and collected in PBS at a density of 2 x 10^4 cells/ml. Cell suspension was mixed with an equal volume of 1% low-melting agarose (Fisher, Fair Lawn, NJ) and placed on agarose-coated slides. Slides were then immersed in lysis solution (1.2 M NaCl, 100 mM Na_2EDTA, 1% Triton X-100, and 0.3nM NaOH, pH 13) at 25° C for 1 h. Slides were rinsed 3 times with alkaline buffer (2 mM Na_2EDTA and 300 mM NaOH) for 20 min each. After electrophoresis in the same alkaline buffer at 20V for 30 min (45), slides were rinsed with distilled water, stained with 2.5 μg/ml of propidium iodide for 20 min, and examined with a Zeiss fluorescence microscope (Thornwood, NY) equipped with an excitation filter of 546 nm and barrier filter of 590 nm. Fifty nuclei per slide were scored for tail moment as a parameter using CometScore software (Tritek, USA).

Western immunoblotting

Cells were lysed in a buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM sodium pyrophosphate, 10% glycerol, 0.1% Na_3VO_4, 50 mM NaF, pH 7.4); cell lysates were isolated from the supernatants after centrifugation of crude lysates at 20,000 *g* for 20 min (7,8). Protein concentration in cell lysates was measured using the BCA assay (Pierce, Rockford, IL). Equal amounts of cellular proteins were resolved by electrophoresis in 10% or 14% SDS-PAGE and transferred to nitrocellulose filters for Western immunoblotting as described previously (9). Antibodies specific to Erk1/2 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific to phosphorylated Erk1/2 (p-Erk1/2), phosphorylated H2AX (p-H2AX), H2AX, phosphorylated Mek1/2 (p-Mek1/2), and Mek1/2 were purchased from Cell Signaling Technology. Antigen-antibody complexes on filters were detected by the Supersignal chemiluminescence kit (Pierce).

Statistical analysis

To statistically verify the cytotoxicity of EC, ECG, EGC, and EGCG, the Shapiro-Wilk test (46) was used to assess normality of cell viability, cell proliferation, and apoptosis. None of the variables showed evidence of lack of normality ($P > 0.05$). Therefore, the Student *t* test was used to compare each of the catechins at each of the concentrations (10, 40, and 100 μg/mL) with the control. Adjustments for multiple comparisons were performed using the Simes method (47).

To statistically verify the suppression of NNK- and B[a]P-induced carcinogenesis by individual catechins, a one-way analysis of variance (ANOVA) test was used to establish significant difference between various treatment groups; a *P* value of ≤ 0.05 was considered

significant. Then, a pairwise analysis of dependent variables was performed with the Duncan multiple range test to verify the significance of differences between groups.

Statistical significance of all the other studies was analyzed by the Student *t* test; α levels were adjusted by the Simes method (47). A *P* value of ≤ 0.05 was considered significant.

Results

NNK- and B[a]P-induced cellular carcinogenesis

Growth factors are required for normal cells to grow and survive, and cell adhesion to extracellular matrixes is important for cell survival in a multi-cell environment; aberrantly-increased cell survivability acquired to reduce dependence on growth factors and to promote anchorage-independent growth can lead cells to tumorigenic transformation (48,49). We used these two important cancer-associated properties as long-term, targeted endpoints for measuring the ability of NNK and B[a]P to induce carcinogenesis of human breast cells. As shown in **Figure 3.1A**, we repeatedly exposed MCF10A cells, for 5, 10, 15, and 20 cycles, to individual or combined NNK and B[a]P each at a bio-achievable concentration of 100 pmol/L, which can be detected in body fluids and tissues of cancer patients and tobacco users (11-13). We detected that accumulated exposures to individual or combined NNK and B[a]P resulted in increasing acquisition of the cancer-associated properties of reduced dependence on growth factors (**Figure 3.1B**) and anchorage-independent growth (**3.1C**) in an exposure-dependent manner, particularly for 5 and 10 cycles. Although accumulated exposures to carcinogens for 20 cycles resulted in higher levels of acquired cancer-associated properties than 10 and 15 exposure cycles, the increased levels appeared to be modest. NNK and B[a]P exhibited comparable abilities to progressively induce breast cell carcinogenesis, and the combination of NNK and B[a]P additively increased degrees of acquired cancer-associated properties. Because cumulative exposures to combined NNK and B[a]P for 10 cycles efficiently and additively induced cellular carcinogenesis versus individual carcinogens, we combined carcinogens each at 100 pmol/L in our extended studies.

Short-term targeted endpoints: ROS elevation, the ERK pathway, cell proliferation, and DNA damage transiently induced by short-term exposure to NNK and B[a]P

To pursue the mechanisms of NNK and B[a]P in inducing breast cell carcinogenesis, we studied the activityof combined NNK and B[a]P each at 100 pmol/L to induce ROS elevation, cell proliferation, DNA damage, and the cell proliferation-related ERK pathway in each short-term exposure. As shown in **Figure 3.2A**, combined NNK and B[a]P at 100 pmol/L induced ROS elevation and cell proliferation. A transient elevation of ROS after NNK and B[a]P treatment was followed by a transient induction of the ERK pathway indexed by phosphorylation of Erk1/2 (**Figure 3.2B**); carcinogen-induced ROS reached its maximal level by 16 h, but Erk1/2 phosphorylation did not reach its maximal level until 24 h. ROS elevation appeared to be induced prior to activation of the ERK pathway during short-term exposure to NNK and B[a]P. To address whether ROS elevation cross-talked with the ERK pathway, we used the antioxidant NAC (28) to block ROS and the Mek1/2 inhibitor U0126 to block the ERK pathway (50). We detected that NAC treatment significantly reduced NNK- and B[a]P-induced, ROS elevation, cell proliferation, and phosphorylation of Mek1/2 and Erk1/2 (**Figure 3.2C**). However, blockage of the ERK pathway did not affect NNK- and B[a]P-induced ROS elevation but reduced cell proliferation (**Figure 3.2D**). These results indicate that NNK- and B[a]P-induced ROS elevation played a role in modulating the ERK pathway for cell proliferation.

To investigate whether NNK- and B[a]P-induced ROS and the ERK pathway were involved in DNA damage, we used a comet assay (51) to detect the extent of nuclear DNA damage by quantifying the DNA damage-produced comet tail moment in agarose gel electrophoresis. We detected that exposure of cells to NNK and B[a]P induced significant DNA damage and H2AX phosphorylation, and blockage of ROS elevation or the ERK pathway

reduced carcinogen-induced DNA damage and H2AX phosphorylation (**Figure 3.2E**), indicating that ROS elevation and ERK pathway activation led to DNA damage during cell exposure to NNK and B[a]P. Thus, cell proliferation, ROS elevation, ERK pathway activation, and DNA damage should be considered as short-term biological, biochemical, and molecular targeted endpoints for measuring the activity of NNK and B[a]P in inducing cellular carcinogenesis.

Cytotoxicity of EC, ECG, EGC, and EGCG

Studies showed that EGCG and EGC are more toxic than ECG and EC in inducing cellular DNA damage (40,41). Whether the cytotoxicity of EC, ECG, EGC, and EGCG contributes to their preventive activity in intervention of cellular carcinogenesis needs to be clarified. To determine the cytotoxicity of individual green tea catechins to breast cells, we investigated the effects of EC, ECG, EGC, and EGCG at various concentrations on viability, proliferation, and apoptotic death of MCF10A cells. As shown in **Figure 3.3**, we detected that none of these catechins at 10 μg/mL showed any detectable effects on reducing cell viability (**3.3A**), inhibiting cell proliferation (**3.3B**), or inducing apoptosis (**3.3C**). EC at 40 μg/mL also failed to induce any detectable cytotoxic effects on MCF10A cells, but EC at 100 μg/mL induced a modest inhibition of cell proliferation and modest apoptosis. ECG, EGC, and EGCG at 40 and 100 μg/mL induced reduction of cell viability (**3.3A**), inhibition of cell proliferation (**3.3B**), and apoptosis (**3.3C**), in a dose-dependent manner. Analysis of these data indicated distinct cytotoxicities of these catechins to MCF10A cells: EC < ECG < EGC < EGCG. At 10 μg/mL, these catechins were non-cytotoxic to MCF10A cells.

Catechin suppression of NNK- and B[a]P-induced short-term targeted endpoints in MCF10A cells

To detect whether individual catechins EC, ECG, EGC, and EGCG were able to counteract against NNK and B[a]P, we studied the activity of these catechins to block NNK- and B[a]P-increased, short-term targeted endpoints. We exposed MCF10A cells to NNK and B[a]P in the presence and absence of individual catechins at 10 μg/mL for 24 h. As shown in **Figure 3.4**, co-treatment with EC, ECG, EGC, and EGCG reduced the NNK- and B[a]P-induced ROS elevation (**3.4A**), Erk1/2 phosphorylation (**3.4B**), cell proliferation (**3.4C**), and DNA damage (**3.4D**) as well as H2AX phosphorylation (**3.4E**). Apparently, these catechins, at non-cytotoxic concentrations, were able to suppress ROS elevation, the ERK pathway, cell proliferation, and DNA damage in non-cancerous breast MCF10A cells. ECG appeared to be more effective than other catechins in reducing these NNK- and B[a]P-induced, short-term biological, biochemical, and molecular targeted endpoints.

Catechin suppression of NNK- and B[a]P-induced short-term targeted endpoints in MCF7 cells

To investigate whether catechin suppression of the NNK- and B[a]P-induced, short-term targeted endpoints of ROS elevation, ERK pathway activation, cell proliferation, and DNA damage was or was not limited to MCF10A cells, we treated human breast cancer MCF7 cells to NNK and B[a]P in the absence and presence of individual catechins for 24 h. We detected that co-treatment with EC, ECG, EGC, and EGCG reduced the NNK- and B[a]P-induced ROS elevation (**Figure 3.5A**), ERK1/2 phosphorylation (**3.5B**), cell proliferation (**3.5C**), and H2AX

phosphorylation (**3.5D**). These results indicated that exposure to NNK and B[a]P also induced short-term targeted endpoints in breast cancer cells. Catechins, at non-cytotoxic levels, were also able to suppress NNK- and B[a]P-induced short-term targeted endpoints in cancer MCF7 cells; ECG appeared to be more effective than other catechins. The results indicate that the ability of NNK and B[a]P to induce short-term targeted endpoints and the activity of catechin in suppression of the NNK- and B[a]P-induced short-term targeted endpoints was not limited to MCF10A cells.

Catechin suppression of chronically NNK- and B[a]P-induced carcinogenesis

To investigate the preventive activity of individual catechins in intervention of breast cell carcinogenesis chronically induced by cumulative exposures to NNK and B[a]P, we repeatedly exposed MCF10A cells to NNK and B[a]P in the absence and presence of individual EC, ECG, EGC, and EGCG at 10 μg/mL for 10 cycles, resulting in the NB, NB/EC, NB/ECG, NB/EGC, and NB/EGCG cell lines, respectively. In addition to the cancer-associated properties of reduced dependence on growth factors and anchorage-independent growth, we also investigated the cancer-associated property of increased cell mobility (52) as the third long-term targeted endpoint to measure carcinogen-induced and catechin-suppressed cellular carcinogenesis. We detected that EC, ECG, EGC, and EGCG effectively suppressed NNK- and B[a]P-induced cellular acquisition of reduced dependence on growth factors (**Figure 3.6A**) as well as anchorage-independent growth (**3.6B**). Not only the numbers but also sizes of colonies were suppressed by catechins (**3.6A** and **3.6B**). The results also revealed that ECG exhibited higher activity than other catechins in suppression of carcinogen-induced cellular acquisition of these

two cancer-associated properties. Using the scratch/wound assay (42), we detected that NB cells acquired higher mobility than parental MCF10A cells to heal the wounded areas, and NB/EC and NB/EGCG cells exhibited higher mobility than NB/ECG and NB/EGC cells to heal the wounded areas (**Figure 3.6C**). Analysis of healing rates revealed that NB/ECG cells exhibited a lower mobility than NB, NB/EGC, NB/EC, and NB/EGCG cells (**Figure 3.6D**), indicating that ECG possessed higher activity than other catechins in suppression of carcinogen-induced cellular acquisition of increased mobility. The high effectivity of ECG in suppressing cellular carcinogenesis was correlated with its activity in blocking ROS elevation and ERK pathway activation, which were transiently-induced by short-term exposure to NNK and B[a]P.

To validate the contributing roles of ROS elevation and the ERK pathway in cellular carcinogenesis induced by cumulative exposures to NNK and B[a]P, we repeatedly exposed MCF10A cells to NNK and B[a]P in the presence of NAC to block ROS, or U0126 to block the ERK pathway for 10 cycles. We detected that blockage of ROS elevation or ERK pathway activation during each cycle of exposure resulted in significant suppression of NNK- and B[a]P-induced cellular acquisition of reduced dependence on growth factors and anchorage-independent growth (**Figure 3.6E**), verifying the contributing roles of ROS elevation and ERK pathway activation in NNK- and B[a]P-induced cellular carcinogenesis.

Discussion

Our model system addresses breast cell carcinogenesis induced by chronic exposure to carcinogens at bio-achievable levels and identifies preventive agents, at non-cytotoxic levels, capable of suppressing chronically-induced breast cell carcinogenesis. We demonstrated, for the first time, a two-step strategy. The first step initially uses short-term biological, biochemical, and molecular targeted carcinogenic endpoints transiently induced by short-term exposure to carcinogens for detecting preventive agents capable of blocking cellular carcinogenesis. The second step subsequently uses long-term biological, biochemical, and molecular targeted carcinogenic endpoints induced by chronic exposure to carcinogens to verify preventive agents effective in suppression of cellular carcinogenesis.

Our studies revealed that cumulative exposures to NNK and/or B[a]P at a bio-achievable concentration of 100 pmol/L resulted in progression of human breast cells to increasingly acquire cancer-associated properties in an exposure-dependent manner, without acquiring tumorigenicity. Although cellular acquisition of tumorigenicity is regarded as the gold standard for validating cell malignancy, many human cancer cells are not tumorigenic, such as MDA-MB-453 (53) and urinary bladder cancer J82 cells (54). Previously, we showed that cumulative exposures of MCF10A cells to NNK and B[a]P at 100 pmol/L for 20 cycles induce the cancer-associated property of acinar-conformational description with irregular spheroids developed on Matrigel (9). Acinar structures with a hollow lumen and apicobasally polarized cells are important characteristics found in glandular epithelia in vivo; the disruption of an intact glandular structure is a hallmark of epithelial cancer, even at its earliest premalignant stages, such as ductal carcinoma *in-situ* (DCIS) (55-57). Clinically, breast cells involved in DCIS are not malignant and have not acquired the ability to invade adjacent tissues through the ductal or

lobular wall, but often premalignant cells can either develop into malignant cells or increase the risk of becoming malignant (4,58,59). Thus, in addition to tumorigenicity, using various cancer-associated properties as measurable targeted endpoints should be seriously considered in studying cellular carcinogenesis and intervention of cellular carcinogenesis.

Short-term exposure to NNK and B[a]P at 100 pmol/L induced transient ROS elevation leading to ERK pathway activation, cell proliferation, and chromosomal DNA damage. However, these short-term targeted endpoints were transient; they were not permanent in cells acquiring cancer-associated properties induced by cumulative exposures to NNK and B[a]P. In our previous studies, we detected that the ERK pathway is downregulated in two individual cell clones isolated from cultures after long-term exposure to NNK (6), and cell proliferation is not increased in cultures after long-term exposure to NNK and/or B[a]P (7-9). Aiming at these transiently-induced, short-term targeted endpoints, we detected green tea catechins EC, ECG, EGC, and EGCG, at a non-cytotoxic concentration of 10 μg/mL, were capable of blocking NNK- and B[a]P-induced ROS elevation, ERK pathway activation, cell proliferation, and DNA damage to various extents in not only non-cancerous MCF10A, but also in adenocarcinoma MCF7 cells. Comparing these catechins at 10 μg/mL revealed that ECG (22.6 μmol/L) was more effective than EC (34.5 μmol/L), EGC (32.7 μmol/L), and EGCG (21.8 μmol/L) to block these short-term targeted carcinogenic endpoints. Interestingly, ECG was also more effective than EC, EGC, and EGCG in suppression of cellular carcinogenesis, measured by degrees of acquired cancer-associated properties in long-term targeted carcinogenic endpoints, permanently induced by cumulative exposures to NNK and B[a]P; however, whether the activity of individual catechins may vary in suppression of cellular carcinogenesis induced by other carcinogens remains to be addressed. In addition, ECG was less cytotoxic to MCF10A cells than EGCG and EGC. Studies

of the hepatotoxicity of GTCs in rats showed that EGCG is the most toxic of all four catechins; for example, the LD_{50} for EGCG (200 μM) was 10 times lower than that for ECG (2000 μM), indicating that ECG is much less toxic than EGCG (60). Thus, the cytotoxicity-independent ability of these catechins to block NNK- and B[a]P-induced, ROS elevation, ERK pathway activation, cell proliferation, and DNA damage in each short-term exposure accounted for their effectiveness in suppression of breast cell carcinogenesis induced by long-term, accumulated exposures to NNK and B[a]P. In addition, in our previous studies, we detected that the NNK- and B[a]P-bioactivating, cytochrome P-450 enzymes CYP1A1 and CYP1B1 are elevated in cells exposed to NNK and B[a]P, and grape seed proanthocyanidin extract is able to reduce these activating enzymes (9). However, we did not detect any activity of GTCs in suppressing NNK- and B[a]P-induced CYP1A1 or CYP1B1 (data not shown).

NNK- and B[a]P-induced ROS elevation played a key role in activation of the ERK pathway, leading to cell proliferation and DNA damage. Studies have shown that ROS is able to induce the ERK pathway via activation of membrane-associated growth factor receptors or via Raf-independent Mek1/2 activation (61,62). In our studies, exposure of MCF10A cells to NNK and B[a]P did not induce any detectable upregulation of Ras or Raf, both of which are upstream from Mek1/2 and Erk1/2 (data not shown). Thus, NNK- and B[a]P-elevated ROS induced the ERK pathway in a Raf-independent manner. However, how ROS was induced by NNK and B[a]P in MCF10A and MCF7 cells, and how ROS was able to induce Raf-independent activation of Mek1/2 and Erk1/2 remain to be determined. Prevention of human breast cell carcinogenesis associated with chronic exposure to low doses of environmental carcinogens is an under-investigated area. Our model system presents unique features of mimicking chronically-induced carcinogenesis of human breast cells to increasingly acquire cancer-associated properties induced

by chronic, cumulative exposures to carcinogens at low concentrations in the pico-molar range, as in environmental exposure. In contrast, many cell systems have been developed to study the activity of carcinogens at high concentrations in the micro-molar range, as in occupational exposure, in acute induction of cellular carcinogenesis (2-5,18-21). Using our cellular model as a target system, we are able to verify the preventive activity of individual green tea catechins at non-cytotoxic levels in suppression of chronic cellular carcinogenesis and identify the mechanisms for catechins in counteracting the biological, biochemical, and molecular effects of NNK and B[a]P. Use of non-cytotoxic catechin components should be seriously considered in prevention of cellular carcinogenesis induced by chronic exposure to environmental carcinogens. Using our model system will conceivably accelerate the identification of additional preventive agents that are effective in reducing the health risk of sporadic breast cancer associated with chronic exposure to carcinogens present in environmental pollution.

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APPENDIX

Figure. 3.1. NNK- and B[a]P-induced cellular carcinogenesis. (**A**) MCF10A cultures were repeatedly exposed to individual (N, B) or combined NNK and B[a]P (NB) each at 100 pmol/L for 5, 10, 15, and 20 cycles. (B) To determine cellular acquisition of the cancer-associated property of reduced dependence on growth factors (RDGF), cells were maintained in LM medium for 10 days. Cell colonies (≥ 0.5 mm diameter) were counted. (**C**) To determine cellular acquisition of the cancer-associated property of anchorage-independent growth (AIG), cells were seeded in soft-agar for 20 days. Cell colonies (≥ 0.1 mm diameter) were counted. Relative degrees of the cancer-associated properties of RDGF and AIG acquired by carcinogen-exposed cells were determined by normalizing with the colony numbers of vehicle-treated counterpart MCF10A cells, set as 1 (X, arbitrary unit), in each exposure cycle. *Columns*, mean of triplicates; *bars*, SD. All results are representative of at least 3 independent experiments. Mean colony numbers in each treatment group were analyzed by one-way ANOVA at $P < 0.05$ to indicate significant difference in number of colonies in various groups. To further determine the significant difference between individual groups, a pairwise analysis of variables was performed using the Duncan multiple range test. Columns with different superscript letters (a, b, c, d, e, f, and g) indicate significant difference at $P < 0.05$ between groups; no significant difference was seen between groups with the same superscript.

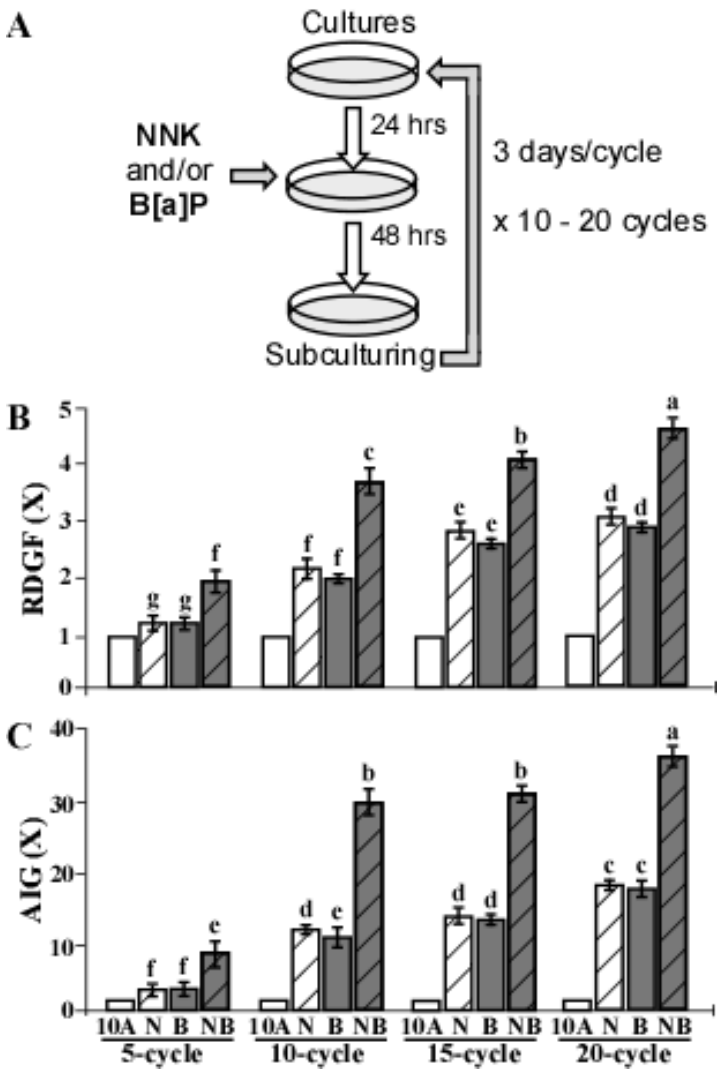


Figure. 3.1.

NNK- and B[a]P-induced cellular carcinogenesis.

Figure. 3.2. Short-term targeted endpoints: ROS elevation, the ERK pathway, cell proliferation, and DNA damage transiently induced by short-term exposure to NNK and B[a]P. (A) MCF10A cells were exposed to combined NNK and B[a]P (NB) each at 100 pmol/L for 24 h. (B) MCF10A cells were exposed to NB for 0, 4, 8, 16, 24, and 48 h. (**C, D,** and **E**) MCF10A cells were exposed to NB in the presence or absence of 5 mmol/L NAC or 10 μmol/L U0126 (U0) for 24 h. (**A, B, C,** and **D**) ROS levels were measured with CM-H_2DCF-DA labeling; relative level of ROS as fold induction was normalized by the level determined in untreated counterpart cells, set as 1 (X, arbitrary unit). (**A, C,** and **D**) Cell proliferation was determined; relative cell growth rate was normalized by the value of BrdU detected in untreated counterpart cells, set as 1 (X, arbitrary unit). (**B, C, D,** and **E**) Cell lysates were prepared and analyzed by Western immunoblotting to detect levels of phosphorylated Mek1/2 (p-Mek1/2), Mek1/2, p-Erk1/2, Erk1/2, p-H2AX, and H2AX, with β-actin as a control, and these levels were quantified by densitometry. The levels of specific phosphorylation of Mek1/2 (p-Mek), Erk1/2 (p-Erk), and H2AX (p-H2AX) were calculated by normalizing the levels of p-Mek1/2, p-Erk1/2, and p-H2AX with the levels of Mek1/2, Erk1/2, and H2AX, respectively, and then further normalizing with the β-actin level and the level set in untreated cells (lane1) as 1 (X, arbitrary unit). (E) DNA damage was measured by a comet assay in 50 cells per treatment; relative DNA damage was normalized by the value determined in untreated counterpart cells, set as 1 (X, arbitrary unit). Representative images of cells treated in the comet assay are shown. *Columns*, mean of triplicates; *bars*, SD. All results are representative of at least 3 independent experiments. The Student *t* test was used to analyze statistical significance, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; α levels were adjusted by the Simes method.

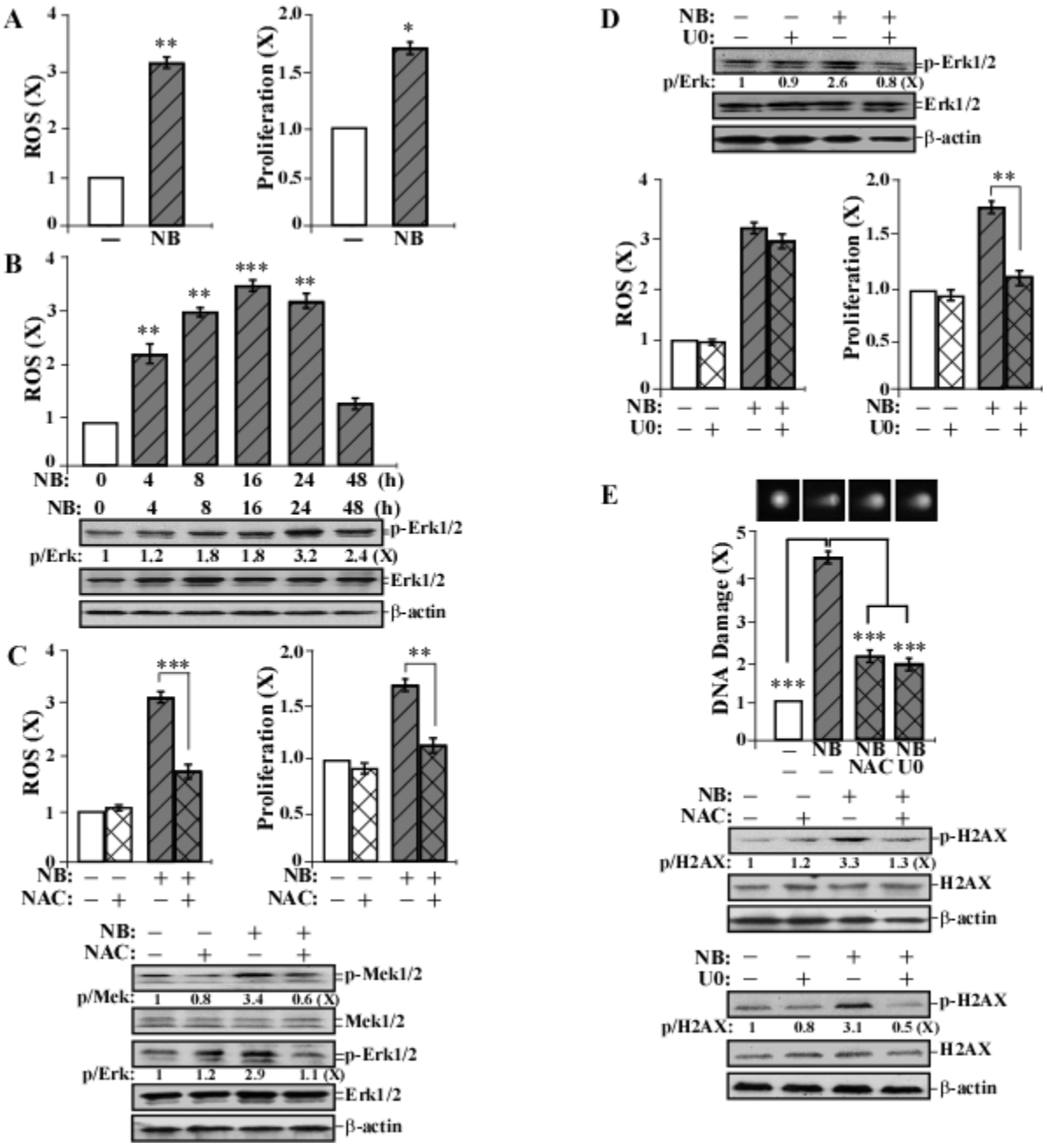


Figure. 3.2.

Short-term targeted endpoints: ROS elevation, the ERK pathway, cell proliferation, and DNA damage transiently induced by short-term exposure to NNK and B[a]P.

Figure. 3.3. Cytotoxicity of EC, ECG, EGC, and EGCG. MCF10A cells were treated with 0, 10, 40 and 100 µg/mL of EC, ECG, EGC, and EGCG individually for 48 h. (A) Quantification of cell viability was determined with an MTT assay kit, and relative cell viability was normalized by the value determined in untreated counterpart cells, set as 100%. (B) Cell proliferation was determined; relative cell growth rate was normalized by the value of BrdU detected in untreated cells, set as 100%. (**C**) Apoptotic cell population (%) was measured by flow cytometry with an Annexin-V-FITC Apoptosis Detection Kit. *Columns*, mean of triplicates; *bars*, SD. All results are representative of at least 3 independent experiments. The Student *t* test was used to analyze statistical significance, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; α levels were adjusted by the Simes method.

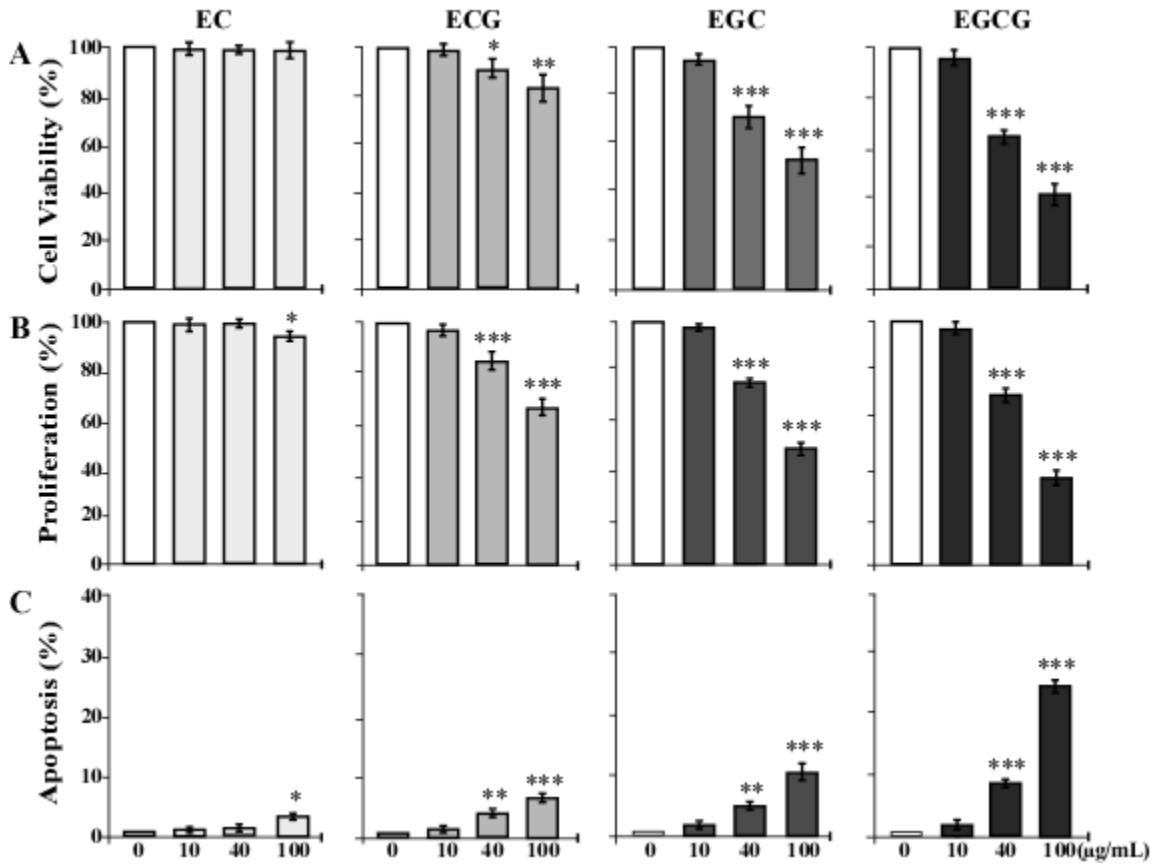


Figure. 3.3.

Cytotoxicity of EC, ECG, EGC, and EGCG.

Figure. 3.4. Catechin suppression of NNK- and B[a]P-induced short-term targeted endpoints in MCF10A cells. MCF10A cells were exposed to combined NNK and B[a]P (NB) each at 100 pmol/L in the absence or presence of 10 μg/mL of EC, ECG, EGC, and EGCG for 24 h. (A) ROS levels were measured with CM-H_2DCF-DA labeling; relative level of ROS as fold induction was normalized by the level determined in untreated counterpart cells, set as 1 (X, arbitrary unit). (**B** and **E**) Cell lysates were prepared and analyzed by Western immunoblotting to detect levels of p-Erk1/2, Erk1/2, p-H2AX, and H2AX, with β-actin as a control, and these levels were quantified by densitometry. The levels of specific phosphorylation of Erk1/2 (p-Erk) and H2AX (p-H2AX) were calculated by normalizing the levels of p-Erk1/2 and p-H2AX with the levels of Erk1/2 and H2AX, respectively, and then further normalizing with β-actin level and the level set in untreated cells (lane1) as 1 (X, arbitrary unit). (**C**) Cell proliferation was determined; relative cell growth rate was normalized by the value of BrdU detected in untreated counterpart cells, set as 1 (X, arbitrary unit). (D) DNA damage was measured by comet assay; relative DNA damage was normalized by the value determined in untreated counterpart cells, set as 1 (X, arbitrary unit). *Columns*, mean of triplicates; *bars*, SD. All results are representative of at least 3 independent experiments. The Student *t* test was used to analyze statistical significance, indicated by ** $P < 0.01$, *** $P < 0.001$; α levels were adjusted by the Simes method.

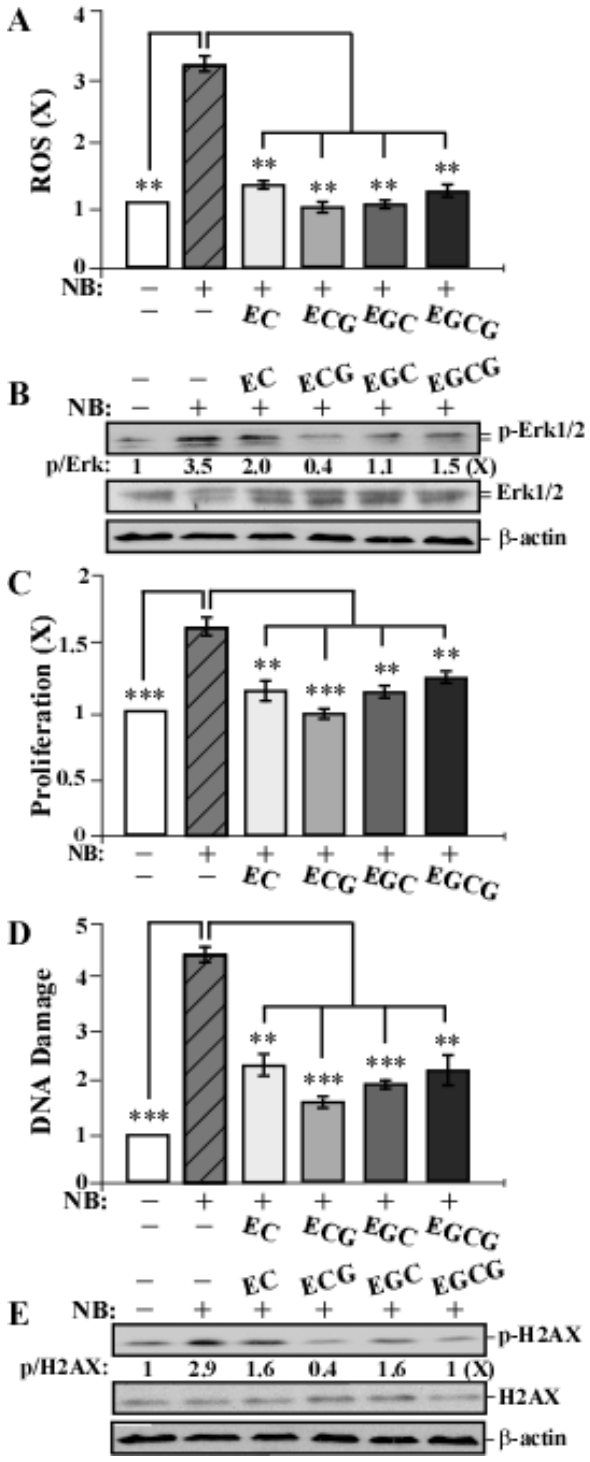


Figure. 3.4.

Catechin suppression of NNK- and B[a]P-induced short-term targeted endpoints in MCF10A cells.

Figure. 3.5. Catechin suppression of NNK- and B[a]P-induced short-term targeted endpoints in MCF7 cells. MCF7 cells were exposed to combined NNK and B[a]P (NB) each at 100 pmol/L in the absence or presence of 10 μg/mL of EC, ECG, EGC, and EGCG for 24 h. (**A**) ROS levels were measured with CM-H_2DCF-DA labeling; relative level of ROS as fold induction was normalized by the level determined in untreated counterpart cells, set as 1 (X, arbitrary unit). (**B** and **D**) Cell lysates were prepared and analyzed by Western immunoblotting to detect levels of phosphorylated Erk1/2 (p-Erk1/2), Erk1/2, p-H2AX, and H2AX, with β-actin as a control, and these levels were quantified by densitometry. The levels of specific phosphorylation Erk1/2 (p-Erk) and H2AX (p-H2AX) were calculated by normalizing the levels of p-Erk1/2 and p-H2AX with the levels of Erk1/2 and H2AX respectively, and then further normalizing with β-actin level and the level set in untreated cells (lane1) as 1 (X, arbitrary unit). (C) Cell proliferation was determined; relative cell growth rate was normalized by the value of BrdU detected in untreated counterpart cells, set as 1 (X, arbitrary unit). *Columns*, mean of triplicates; *bars*, SD. All results are representative of at least 3 independent experiments. The Student *t* test was used to analyze statistical significance, indicated by ** $P < 0.01$, *** $P < 0.001$; α levels were adjusted by the Simes method.

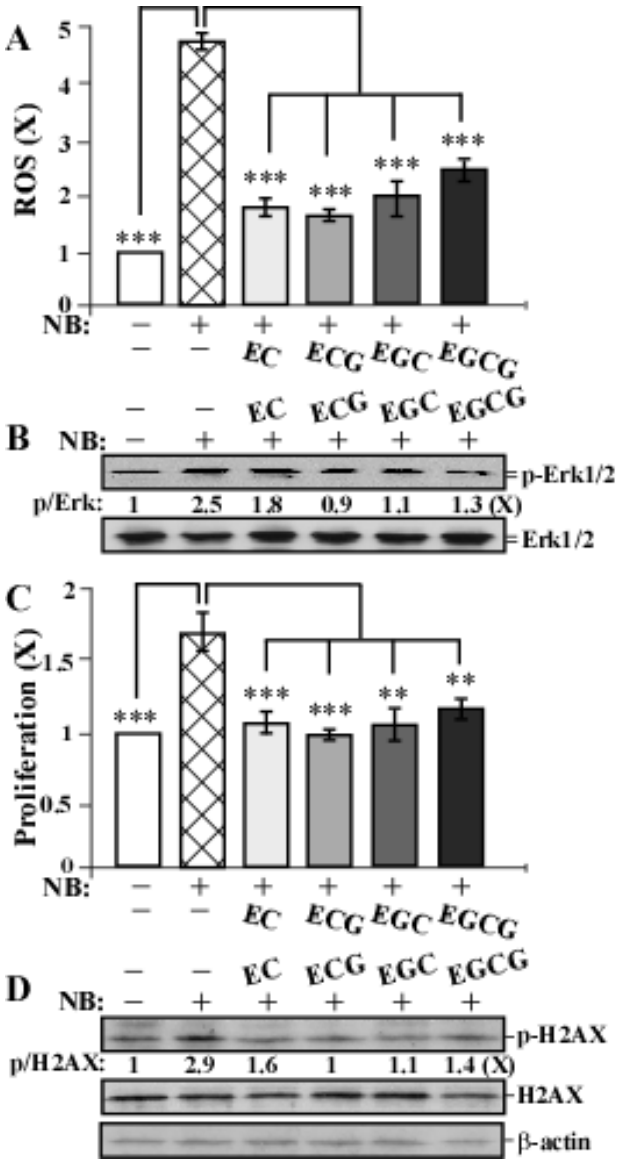
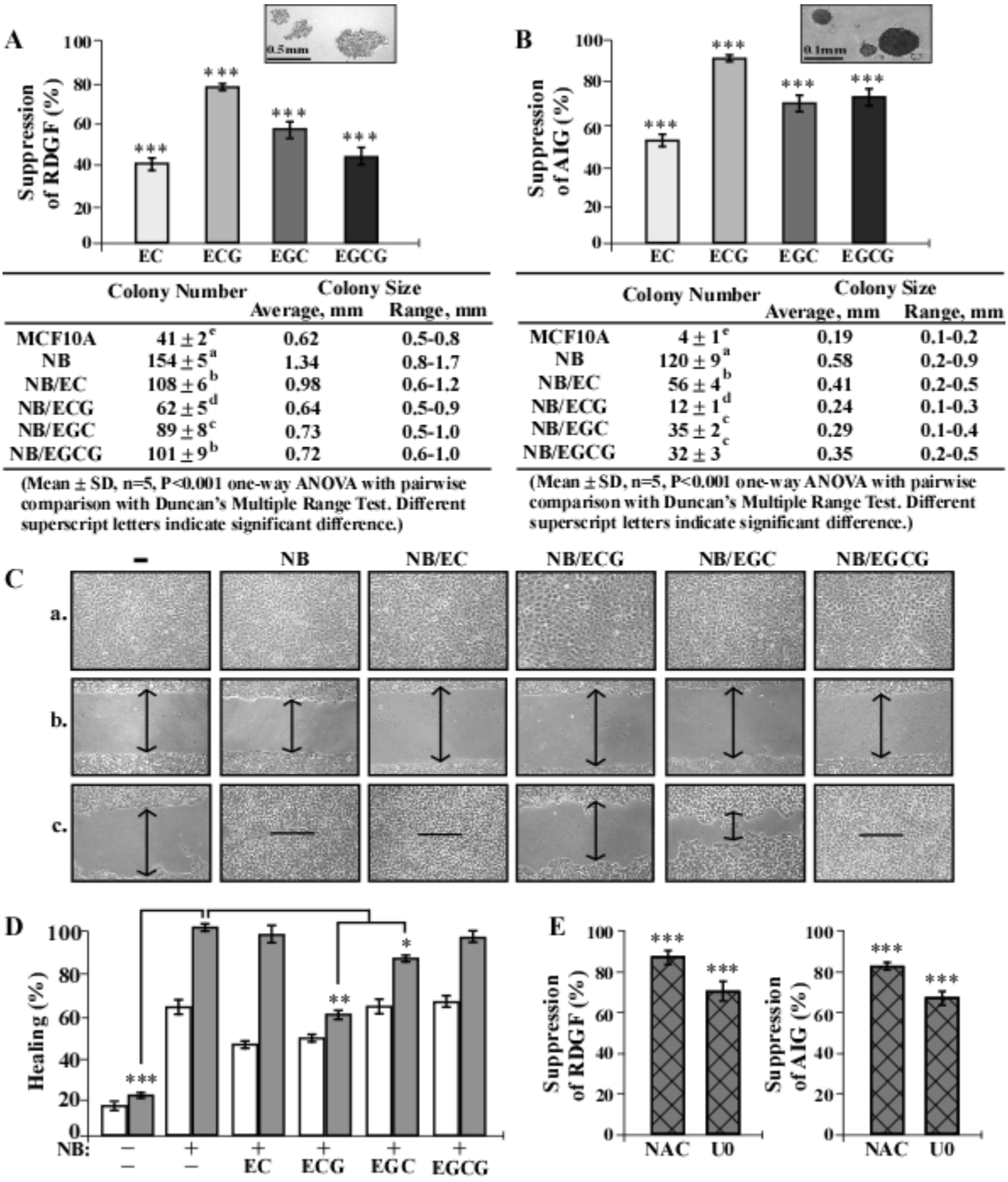


Figure. 3.5.

Catechin suppression of NNK- and B[a]P-induced short-term targeted endpoints in MCF7 cells.

Figure. 3.6. Catechin suppression of chronically NNK- and B[a]P-induced carcinogenesis. (**A-D**) MCF10A cultures were repeatedly exposed to combined NNK and B[a]P (NB) each at 100 pmol/L in the presence of 10 μg/mL of EC, ECG, EGC, and EGCG (NB/catechin) for 10 cycles. (**E**) MCF10A cultures were repeatedly exposed to NB in the presence of 5 mmol/L NAC or 10 μmol/L U0126 (U0) for 10 cycles. (A and E) To detect effectivity of individual catechins, NAC, and U0 on suppression of cellular acquisition of reduced dependence on growth factors (RDGF), 5×10^3 cells were seeded and maintained in LM medium for 10 days. (**B** and **E**) To detect effectivity of individual catechins, NAC, or U0 on suppression of cellular acquisition of anchorage-independent growth (AIG), 1×10^4 cells were seeded in soft-agar for 20 days. The value of the suppression effectivity of individual catechins on NB-induced RDGF (**A** and **E**) and AIG (**B** and **E**) was calculated by: {1 − [(# of NB/catechin-induced cell colonies) − (# of MCF10A cell colonies)] ÷ [(# of NB-induced cell colonies) − (# of MCF10A cell colonies)]} x 100 (%). The Student *t* test was used to analyze statistical significance, indicated by *** $P < 0.001$; α levels were adjusted by the Simes method. Tables show colony numbers, average colony size, and range of colony size. Mean colony numbers in each treatment group were analyzed by one-way ANOVA at $P < 0.001$ to indicate significant difference in number of colonies in various groups. To further determine the significant difference between individual groups, a pairwise analysis of variables was performed using the Duncan multiple range test. Means with different superscript letters (a, b, c, d, and e) indicate significant difference at $P < 0.001$ between groups; no significant difference was seen between groups with the same superscript. (**C**) To detect effectivity of individual catechins in suppressing carcinogen-induced cellular acquisition of increased mobility, cells were seeded in CM medium and grown to confluence (**a**), a linear area of cell layer was removed from each culture with a 23-gauge needle

to produce wounded cultures, and the wounded areas were examined (×100 magnification) 6 (**b**) and 24 h (**c**) after wounding. Arrows indicate width of wounded areas. Results are representative of 3 independent experiments. (**D**) To quantitatively measure cell mobility detected in (**C**), the area not healed by the cells was subtracted from total area of initial wound to calculate the wound healing area (%) at time intervals of 6 h (white columns) and 24 h (grey columns). *Columns*, mean of triplicates; *bars*, SD. All results are representative of 3 independent experiments. The Student *t* test was used to analyze statistical significance, indicated by * $P < 0.05$, ** $P < 0.01$; α levels were adjusted by the Simes method.



	Colony Number	Colony Size Average, mm	Colony Size Range, mm
MCF10A	41 ± 2^{e}	0.62	0.5-0.8
NB	154 ± 5^{a}	1.34	0.8-1.7
NB/EC	108 ± 6^{b}	0.98	0.6-1.2
NB/ECG	62 ± 5^{d}	0.64	0.5-0.9
NB/EGC	89 ± 8^{c}	0.73	0.5-1.0
NB/EGCG	101 ± 9^{b}	0.72	0.6-1.0

(Mean ± SD, n=5, P<0.001 one-way ANOVA with pairwise comparison with Duncan's Multiple Range Test. Different superscript letters indicate significant difference.)

	Colony Number	Colony Size Average, mm	Colony Size Range, mm
MCF10A	4 ± 1^{e}	0.19	0.1-0.2
NB	120 ± 9^{a}	0.58	0.2-0.9
NB/EC	56 ± 4^{b}	0.41	0.2-0.5
NB/ECG	12 ± 1^{d}	0.24	0.1-0.3
NB/EGC	35 ± 2^{c}	0.29	0.1-0.4
NB/EGCG	32 ± 3^{c}	0.35	0.2-0.5

(Mean ± SD, n=5, P<0.001 one-way ANOVA with pairwise comparison with Duncan's Multiple Range Test. Different superscript letters indicate significant difference.)

Figure. 3.6.

Catechin suppression of chronically NNK- and B[a]P-induced carcinogenesis.

PART –IV

Mesenchymal and stem-like cell properties targeted by green tea and grape seed extract in suppression of chronic breast cell carcinogenesis

Research described in this chapter is submitted for publication in Molecular Carcinogenesis by Kusum Rathore and Hwa-Chain Robert Wang

Kusum Rathore and Hwa-Chain Robert Wang. Mesenchymal and Stem-Like Cell Properties Targeted by Green Tea and Grape Seed Extract in Suppression of Chronic Breast Cell Carcinogenesis
(Molecular Carcinogenesis -Submitted)

In this paper “our” and “we” refers to me and co-authors. My contribution in the paper includes (1) Selection of the topic (2) Compiling and interpretation of the literature (3) Designing experiments (4) understanding the literature and interpretation of the results (5) providing comprehensive structure to the paper (6) Preparation of the graphs and figures (7) Writing and editing

Abstract

Cancer stem-like cells and the epithelial-to-mesenchymal transition (EMT) are postulated to play roles in various stages of cancer development, but their roles in chronic breast cell carcinogenesis remain to be clarified. Here, we investigated the roles of properties and markers associated with stem-like cells and the EMT in carcinogenesis chronically induced by chemical carcinogens, as well as their roles in intervention of carcinogenesis by dietary components. We repeatedly treated immortalized, non-cancerous, human breast epithelial MCF10A cells with pico-molar concentrations of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a]P) in culture to progressively induce acquisition of cancer-associated properties, stem-like cell- and EMT-associated properties and markers. We used dietary green tea catechins (GTC) and grape seed proanthocyanidin extract (GSPE), at non-cytotoxic concentrations, in intervention of these associated properties and markers. We detected that cumulative exposures to low doses of NNK and B[a]P resulted in cellular acquisition of stem-like cell- and EMT-associated properties and markers in addition to cancer-associated properties. The stem-like cell-associated properties and markers included increases in mammosphere formation, and in aldehyde dehydrogenase-positive and $CD44^{+}/CD24^{-}$ cell populations. The EMT-associated properties and markers included mesenchymal cell morphology; increased cell migration, invasion, and mobility; and changed expression of E-Cadherin, EpCAM, Vimentin, and MMP-9. We also detected that non-cytotoxic GTC and GSPE were effective at intervening in cellular acquisition of stem-like cell- and EMT-associated properties and markers induced by NNK and B[a]P. Thus stem-like cell- and EMT-associated properties and markers could be considered as new targets to use in identifying induction and intervention of breast cell carcinogenesis.

Introduction

Carcinogenesis of human breast epithelial cells from non-cancerous to pre-malignant and malignant stages is a multiyear, multistep, and multipath disease process involving cumulative genetic and epigenetic alterations [1, 2]. More than 85% of breast cancers are sporadic and attributable to long-term exposure to environmental factors, such as chemical carcinogens [1-5]. Over 200 chemical carcinogens have been experimentally detected to acutely induce malignancy in breast cells in cultures or mammary tumors in animals [1,3,6]. These carcinogens have been studied at high doses of micro- to milli-molar concentrations [1,3,6], which is appropriate in examining occupational exposure. However, considering that carcinogenesis of human tissues involves long-term exposure to environmental carcinogens at low doses, a high-dose approach may not serve as a proper way to study environmental exposure. Thus, it is imperative to take a chronic, low-dose approach to reveal environmental mammary carcinogens, at physiologically-achievable levels, capable of inducing human breast cell carcinogenesis.

Our chronic carcinogenesis model has revealed that at physiologically-achievable pico-molar concentrations [7-10], two environmental carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a]P) are capable of inducing carcinogenesis of immortalized, non-cancerous, human breast epithelial MCF10A cells [11-15]. NNK is considered one of the most potent lung carcinogens in tobacco products [16]; although gastric administration of NNK into rats results in DNA-adduct formation in the mammary gland and development of mammary tumors [16-19], NNK is not currently recognized as a breast carcinogen. B[a]P, a family member of polycyclic aromatic hydrocarbons, is considered an environmental, dietary, and tobacco carcinogen. Its metabolites form strong DNA adducts and cause DNA lesions, and B[a]P is recognized as a mammary carcinogen in rodents [1,4,10,20-23].

In our model [11-15], 24 h after each subculturing, MCF10A cultures receive 48-h cycles of exposure to NNK and B[a]P. Although in immune-deficient mice, cells repeatedly exposed to 100 pmol/L NNK and B[a]P each for 20 cycles do not form xenograft tumors, the cumulative exposures result in cellular acquisition of progressively-increased degrees of various cancer-associated properties, including reduced dependence on growth factors, anchorage-independent growth, and acinar-conformational disruption in an exposure cycle-dependent manner. Hence, our cell model system, which admits development of measurable cancer-associated properties, is highly valuable for sensitive detection of whether low doses of carcinogens are capable of inducing non-tumorigenic, premalignant carcinogenesis of breast cells.

Using our model, we have detected that non-cytotoxic concentrations of green tea catechins (GTC) and grape seed proanthocyanidin extract (GSPE) are effective, in a dose-dependent manner, in intervention of NNK- and B[a]P-induced cellular carcinogenesis. This effectiveness is measured by their ability to suppress the carcinogen-induced, biological targeted endpoints of reduced dependence on growth factors, anchorage-independent growth, and acinar-conformational disruption [12-15]. GTC has been shown to possess inhibitory and apoptotic activity in human breast cancer cells in cultures [24, 25], and GTC is able to suppress mammary carcinogenesis induced by 7,12-dimethylbenz[a]anthracene and N-methyl-N-nitrosourea in rats [26, 27]. Although the results of epidemiological studies are controversial, some evidence has indicated that green tea consumption may help prevent breast cancer recurrence in early stage cancers [28, 29]. GSPE has been shown to exhibit antioxidant and anticancer activities in both in vitro and in vivo models [30–33], and it shows diet-dependent, chemopreventive activity in suppression of mammary tumors induced by 7,12-dimethylbenz[a]anthracene in rats [33]. Several epidemiological studies suggests benefits of consumption of grapes in prevention of

human cancers, including breast [34], bone [35], and oral cancer [36]. Thus, it is important to further understand the preventive activity of GTC and GSPE in intervention of premalignant, breast cell carcinogenesis chronically induced by environmental carcinogens. Particularly, understanding the preventive activity of non-cytotoxic GTC and GSPE will be highly valuable to targeted intervention of premalignant carcinogenesis to reduce the health risk of sporadic breast cancer.

It has been postulated that cancer stem-like cells are involved in generating and maintaining premalignant and malignant lesions [37-39], and development of stem-like cells may involve induction of the epithelial-to-mesenchymal transition (EMT) program [40]. However, it is not clear whether cancer stem-like cells and the EMT program can be induced by long-term exposure to low doses of carcinogens like that in chronic breast cell carcinogenesis. Whether preventive agents, like GTC and GSPE, are able to suppress the induction of cancer stem-like cells and the EMT program also remains to be clarified.

In this communication, we investigated stem-like cell- and EMT-associated properties and markers progressively induced by chronic exposure of breast epithelial cells to NNK and B[a]P. We also used stem-like cell- and EMT-associated properties and markers as targeted endpoints to verify the activity of non-cytotoxic GTC and GSPE in suppression of NNK- and B[a]P-induced cellular carcinogenesis. Thus, the measurable, stem-like cell- and EMT-associated properties and markers should be considered as new cancer-associated indicators and targeted endpoints for detecting breast cell carcinogenesis and suppression of progression.

Materials and Methods

Cell cultures and reagents

Immortalized, non-cancerous, human breast epithelial MCF10A (American Type Culture Collection [ATCC], Rockville, MD) and derived cell lines were maintained in complete MCF10A (CM) medium (1:1 mixture of DMEM/Ham's F12, supplemented with 100 ng/ml cholera enterotoxin, 10 μg/ml insulin, 0.5 μg/ml hydrocortisol, 20 ng/ml epidermal growth factor, and 5% horse serum) as well as 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37° C [6-9]. All cultures were maintained in 5% CO_2 at 37°C. Stock aqueous solutions of NNK (Chemsyn, Lenexa, KS) and B[a]P (Aldrich, Milwaukee, WI) were prepared in DMSO and diluted with culture medium for assays. GTC (Polyphenon-60, a mixture of polyphenolic compounds containing 60% total catechins, Sigma, St. Louis, MO) and GSPE (contains 74% proanthocyanidins, InterHealth Nutraceuticals, Benicia, CA) were prepared in distilled water and diluted with medium.

Induction and suppression of cell carcinogenesis

Twenty-four hours after each subculturing, MCF10A cells were treated with combined NNK and B[a]P each at 100 pmol/L in the absence and presence of GTC or GSPE for 48 h as one cycle of exposure for 20 cycles; cultures were subcultured every 3 d.

Reduced dependence on growth factors assay

The low-mitogen (LM) medium contained total serum and mitogenic additives reduced to

2% of the concentration formulated in CM medium as described above [12-15]. Five × 10^3 cells were seeded in 60-mm culture dishes and maintained in LM medium. Growing cell colonies that reached 0.5 mm diameter in 10 d were stained with Coomassie brilliant blue and identified as cell clones acquiring the cancer-associated property of reduced dependence on growth factors.

Anchorage-independent cell growth assay

The base layer consisted of 2% low-melting agarose (Sigma-Aldrich) in CM medium. Then, 0.4% low-melting agarose in a mixture (1:1) of CM medium with 3-d conditioned medium prepared from MCF10A cultures was mixed with 1 × 10^4 cells and plated on top of the base layer in 60-mm diameter culture dishes. Growing colonies that reached 0.1 mm diameter by 20 d were identified as cell clones acquiring the cancer-associated property of anchorage-independent growth.

Cell mobility-healing assay

Cells were seeded in 6-well plates and grown to confluence in CM medium. Cells were rinsed with phosphate-buffered saline (PBS) and maintained in DMEM/Ham's F12 medium supplemented with 2% horse serum for 15 h [41]. The monolayer was then scratched with a 23-gauge needle (BD Biosciences, Franklin Lakes, NJ) to generate wounds and rinsed with CM medium to remove floating cells. Cultures were maintained in CM medium, and the wounded areas were examined 6 h and 20 h after scratches to detect healing. Wound healing area was calculated using Total Lab TL100 software (Total Lab, Newcastle, NE).

In vitro cell invasion and migration assay

The *in vitro* cell invasion assay was performed using 24-well Transwell insert-chambers with a polycarbonate filter with a pore size of 8.0 μm (Corning Costar, Lowell, MA). The upper side of the filter was coated with a Matrigel membrane (BD Biosciences). Two × 10^4 cells in serum-free medium were seeded on top of the Matrigel-coated filter in each insert-chamber. Then, insert-chambers were placed into wells on top of culture medium containing 10% horse serum as a chemoattractant. After 24 h, the invasive ability of cells was determined by the number of cells translocated to the lower side of filters.

The *in vitro* migration assay was performed using 24-well Transwell insert-chambers with a polycarbonate filter without Matrigel. The migration ability of cells was determined by the number of cells translocated to the lower side from the upper side of filters [42].

Mammosphere formation

Cells were seeded on 100-mm culture dishes on top of a 1% agarose-coated, non-adherent culture plate, incubated in serum-free, complete MCF10A medium supplemented with 0.4% bovine serum albumin, and maintained in 5% CO_2 at 37° C for 7 to 10 d to develop mammospheres [43].

Aldehyde dehydrogenase (ALDH) assay

An ALDEFLUOR Kit (StemCell Technologies, Vancouver, BC) was used to detect ALDH-expressing cells. One × 10^5 cells/ml were resuspended in assay buffer, mixed with

activated Aldefluor substrate BAAA (BODIPY-aminoacetaldehyde), and incubated in the presence and absence of the ALDH inhibitor diethylaminobenzaldehyde (DEAB) at 37° C for 40 minutes. Then, cells were resuspended in assay buffer for flow cytometric analysis by using a 15 milliwatt air-cooled argon laser to produce 488 nm light [44]. Fluorescence emission was collected with a 529-nm band pass filter. The mean fluorescence intensity of cells was quantified using Multicycle software (Phoenix Flow System, San Diego, CA). Cells incubated with BAAA in the presence of DEAB were used to establish the baseline of fluorescence for determining the ALDH-expressing cell population (%) in which ALDH activity was not inhibited by DEAB.

Flow cytometric detection of CD44 and CD24 cells

Cells were trypsinzed and washed with glycine wash buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM, NaH_2PO_4, and 100 mM glycine). Cells were then suspended in blocking buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM, NaH_2PO_4, 8 mM NaN_3, 0.1% BSA, 0.2% TritonX-100, and 0.05% Tween 20) and incubated with phycoerythrin (PE)-conjugated CD44-specific antibody and fluorescein isothiocyanate (FITC)-conjugated CD24-specific antibody (BD Biosciences) at 4° C for 15 h. Cells were rinsed and suspended in PBS, and 1×10^5 cells/ml was for analyzed by flow cytometry to determine the $CD44^{+/-}$ and $CD24^{+/-}$ cell populations.

Immunofluorescence detection of CD44 and CD24 cells

Mammospheres were collected, washed with glycine wash buffer, and suspended in blocking buffer. They were then incubated with PE-conjugated CD44-specific antibody and FITC-conjugated CD24-specific antibody at 4° C for 15 h. The mammospheres were rinsed with

PBS and 0.1%Tween 20, detected by confocal epifluorescence microscope (Leica TCS SP2, Leica Microsystems Heidelberg, Germany), and analyzed with Leica Lite software (Leica Microsystems).

Western immunoblotting

Cells were lysed in buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM sodium pyrophosphate, 10% glycerol, 0.1% Na_3VO_4, and 50 mM NaF, pH 7.4). Cell lysates were isolated from the supernatants after centrifugation of crude lysates at 20,000 × g for 20 minutes. Protein concentration in cell lysates was measured using the BCA assay (Pierce, Rockford, IL). Equal amounts of cellular proteins were resolved by electrophoresis in either 10% or 14% SDS-polyacrylamide gels and transferred to nitrocellulose filters for Western immunoblotting as previously described [12-15]. Antibodies specific to E-Cadherin, Ep-CAM, MMP-9, Vimentin, and β-Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antigen-antibody complexes on filters were detected by the Supersignal chemiluminescence kit (Pierce).

Statistical analysis

A one-way analysis of variance (ANOVA) test was used to establish significant difference between various treatment groups; a P value of ≤ 0.05 was considered significant. Then, a pairwise analysis of dependent variables was performed with the Duncan multiple range test to verify the significance of differences between groups.

Statistical significance of was analyzed by the Student *t* test; α levels were adjusted by the Simes method [45]. A *P* value of $\leq$ 0.05 was considered significant.

Results

NNK- and B[a]P-induced cellular carcinogenesis

A lack of growth factors causes normal cells to become growth-arrested in the cell cycle and to commit apoptosis; however, reduced dependence on growth factors contributes to aberrantly-increased cell survivability and ultimately to cellular carcinogenesis [46-48]. Cell adhesion, or anchorage, to extracellular matrixes is important for normal cell survival in a multi-cell environment; therefore, anchorage-independent growth also promotes aberrantly-increased cell survivability and contributes to cellular carcinogenesis [49,50]. We have routinely used these two cancer-associated properties of reduced dependence on growth factors and anchorage-independent growth as biological targeted endpoints for measuring the progression of chronic cellular carcinogenesis [12-15]. As demonstrated in previous reports [14-15], cumulative exposures of MCF10A cells to NNK and B[a]P, at physiologically-achievable concentrations of 100 pmol/L, for 5, 10, and 20 cycles (resulting in NB5, NB10, and NB20 cell lines, respectively), induce cells to acquire increased degrees of reduced dependence on growth factors and anchorage-independent growth, as determined by increased numbers of cell clones survived in LM medium and soft agar, respectively. As performed in this report, additional studies revealed that cumulative exposures to NNK and B[a]P resulted in not only increasing the number of cell clones (**Figure 4.1A-1** and **4.1B-1**) but also in increasing the sizes of cell colonies, measured as diameter, (**Figure 4.1A-2** and **4.1B-2**). The tumorigenic, malignant MCF10A-Ras cell line, in which oncogenic H-Ras is ectopically expressed in MCF10A cells [51,52], developed a significantly higher number of clones and had larger cell colonies than the premalignant NB5, NB10, and NB20 cells (**Figure 4.1A-2** and **4.1B-2**). These results indicate that cumulative exposures of breast cells to NNK and B[a]P resulted in increasing acquisition of

cancer-associated properties of reduced dependence on growth factors and anchorage-independent growth.

NNK- and B[a]P-induced stem-like cell properties

It has been shown that stem-like cells are able to self-renew in serum-free medium and develop mammospheres in non-adherent cultures [43]. Considering the stem-like cell property of serum-independent, non-adherent growth, stem-like cells appear to acquire both abilities of reduced dependence on growth factors and anchorage-independent growth that are acquired by cells undergoing carcinogenesis. In addition, cancer stem-like cells have been postulated to play roles in generating and maintaining premalignant and malignant lesions [37-39]. Thus, whether cellular carcinogenesis generates stem-like cells is an important question to be clarified. Using agarose-coated culture plates, we successfully grew mammospheres from non-cancerous MCF10A cells, NNK- and B[a]P-exposed pre-malignant NB cells, and malignant MCF10A-Ras cells. As shown in **Figure 4.2A-1** and **4.2A-2**, accumulated exposures of MCF10A cells to NNK and B[a]P resulted in increased number and size of mammospheres in an exposure-dependent manner, as detected in parental and pre-malignant NB5, NB10, and NB20 cultures; higher numbers and larger sizes of mammospheres developed in MCF10A-Ras cultures than in NB20 and NB10 cultures. Breast stem-like cells have also been shown to exhibit high levels of ALDH activity [44] and express a high level of cell surface marker CD44 with a low level of CD24 [53]. We detected significantly-increased ALDH-positive (**Figure 4.2B**) and $CD44^+/CD24^-$ (**4.2C** and **4.2D**) cell populations in mammospheres developed in NB10, NB20, and MCF10A-Ras cultures; also, higher populations of ALDH-positive and $CD44^+/CD24^-$ cells developed in MCF10A-Ras

cultures than in NB20 and NB10 cultures. Apparently, cumulative exposures to NNK and B[a]P resulted in acquisition of stem-like cell properties that may be seriously considered as a new cancer-associated property to be used to measure progress of cellular carcinogenesis.

NNK- and B[a]P-induced EMT-associated properties and markers

Studies have shown that the EMT program is involved in development of stem-like cells [40]. Induction of the EMT program in epithelial cells results in the cells exhibiting the fibroblastoid morphology of mesenchymal cells with increased invasive ability [54,55]. It has been shown that ectopic expression of oncogenic H-Ras in MCF10A cells induces mesenchymal morphology and EMT-associated markers [56]. In investigating whether chronic NNK and B[a]P exposure resulted in induction of the EMT program, we observed morphologic changes of MCF10A cells from compactly-attached cobblestone-like epithelial morphology to dispersed, spindle-like mesenchymal morphology in NB10 and NB20 cultures, as compared with MCF10A-Ras cells exhibiting typical mesenchymal cell morphology (**Figure 4.3A**). Studying EMT-associated markers, we detected decreased levels of E-Cadherin and EpCAM as well as increased levels of Vimentin and MMP-9 in NB10, NB20, and MCF10A-Ras cells (**Figure 4.3B**); changes of these EMT-associated markers were in concert with the increased degrees of cellular carcinogenesis. During the EMT reduction of E-Cadherin and EpCAM is involved in loses of cell-cell adhesion [57,58], while an increase of MMP-9 is involved in the breakdown of extracellular matrix [59], and increased Vimentin is involved in filament expression and increased mobility [60]. Thus, addressing whether cellular carcinogenesis resulted in acquisition of EMT-associated properties [55], we detected increased levels of the EMT-associated

properties of cell migration and invasion acquired by NNK- and B[a]P-exposed, NB10 and NB20 cells; NB20 acquired higher levels of these properties than NB10 and comparable levels to MCF10A-Ras cells (**Figure 4.3C** and **4.3D**). In addition, using a scratch/wound assay [41], we detected that NB20 cells acquired higher degrees of cell mobility than NB10 and NB5 cells, and NB20 cells acquired a comparable degree of cell mobility to MCF10A-Ras cells (**Figure 4.3E-1** and **4.3E-2**). Accordingly, these results indicate that cumulative exposures to NNK and B[a]P resulted in acquisition of EMT-associated properties in an exposure cycle-dependent manner; thus, EMT-associated properties and markers may be considered as new cancer-associated properties to be used as targeted endpoints to measure progress of cellular carcinogenesis.

GTC and GSPE suppression of NNK- and B[a]P-induced cellular carcinogenesis

Using the cancer-associated properties of reduced dependence on growth factors and anchorage-independent growth as biological targeted endpoints, we investigated the activity of GTC and GSPE in suppression of NNK- and B[a]P-induced cellular carcinogenesis. Consistent with our reported results [14-15], GTC and GSPE at their maximal, non-cytotoxic concentration of 40 μg/mL were effective in suppressing the number of cell clones acquiring reduced dependence on growth factors and anchorage-independent growth (**Figure 4.4A-1** and **4.4B-1**). Cumulative exposures of MCF10A cells to NNK and B[a]P in the presence of GTC and GSPE for 20 cycles resulted in NB/T-20 and NB/G-20 cell lines, respectively. Here, we performed additional studies to reveal that co-exposure to GTC and GSPE resulted in not only decreasing the number of cell clones (**Figure 4.4A-1** and **4.4B-1**) but also in decreasing cell abilities of reduced dependence on growth factors (**4.4A-2**) and anchorage-independent growth (**4.4B-2**), as

determined by decreased sizes of cell colonies in NB/T-20 and NB/G-20 cultures versus NB20 cultures. These results indicate that non-cytotoxic GTC and GSPE were able to qualitatively and quantitatively suppress NNK- and B[a]P-induced cellular carcinogenesis.

GTC and GSPE suppression of NNK- and B[a]P-induced stem-like cell properties

Using the stem-like cell properties of mammosphere formation and increased ALDH-positive and $CD44^+/CD24^-$ cell populations as targeted endpoints, we furthered our understanding of the activity of GTC and GSPE in suppression of NNK- and B[a]P-induced cellular carcinogenesis. As shown in **Figure 4.5A**, co-exposure to GTC and GSPE significantly reduced not only the number but also the size of mammospheres induced by NNK and B[a]P. Co-exposure to GTC and GSPE also significantly reduced ALDH-positive (**Figure 4.5B**) and $CD44^+/CD24^-$ (**4.5C**) cell populations in mammospheres, as determined in NB/T-20 and NB/G20 cultures versus NB20 cultures. These results indicate that non-cytotoxic GTC and GSPE were able to suppress NNK- and B[a]P-induced stem cell-like properties.

GTC and GSPE suppression of NNK- and B[a]P-induced EMT-associated properties and markers

Using EMT-associated cell morphology, markers and properties as targeted endpoints, we investigated the activity of GTC and GSPE in suppression of NNK- and B[a]P-induced cellular carcinogenesis. As shown in **Figure 4.6A**, mesenchymal-like cell morphology in NB20 cultures was not detected in NB/T-20 and NB/G20 cultures. Expression of E-Cadherin and EpCAM was reduced in NB20 cells but not in NB/T-20 and NB/G20 cells, and increased MMP-9 and Vimentin in NB20 cells were reversed in NB/T-20 and NB/G20 cells (**Figure 4.6B**). In

addition, the EMT-associated properties of increased cell migration (**Figure 4.6C**), invasion (**4.6D**), and mobility (**4.6E-**1 and **4.6E-2**) acquired by NB20 cells were reversed in in NB/T-20 and NB/G20 cells. These results indicate that non-cytotoxic GTC and GSPE were able to protect epithelial cells from developing NNK- and B[a]P-induced EMT-associated morphological features, markers, and properties.

Discussion

Cumulative exposures of non-cancerous human breast epithelial MCF10A to the carcinogens NNK and B[a]P, at physiologically-achievable concentrations, result in progression of cellular carcinogenesis to premalignant stages. This progression is measured by cellular acquisition of progressively-increased degrees of cancer-associated properties in an exposure-dependent manner, without acquiring tumorigenicity [11-15]. Although cellular acquisition of tumorigenicity is regarded as the gold standard for validating cell malignancy, many human cancer cells are not tumorigenic, such as urinary bladder cancer J82 cells [61]. Thus, using various other cancer-associated properties as measurable targeted endpoints should be seriously considered in studying cellular carcinogenesis progression and intervention of cellular carcinogenesis.

In this study, we presented results to reveal the values of acquisition of stem-like cell properties as well as EMT-associated markers and properties in measuring the induction of chronic cellular carcinogenesis. Epithelial cells chronically exposed to NNK and B[a]P increasingly acquired stem-like cell properties of mammosphere formation and increased

populations of ADLH-positive and $CD44^+/CD24^-$ stem-like cells in mammospheres. The ability of carcinogen-exposed epithelial cells to develop mammospheres in serum-free non-adherent cultures may be related to their combined abilities of reduced dependence on growth factors and anchorage-independent growth. The ability of carcinogenic epithelial cells to produce increased stem-like cell populations in mammospheres may be mediated by the EMT program, as indicated by many studies [43,56,62]. Our results revealed that the EMT-associated properties of mesenchymal morphology, cell migration, invasion, and mobility, as well as the EMT-associated markers of losing E-Cadherin and EpCAM and gaining MMP-9 and Vimentin were also increasingly acquired by epithelial cells chronically exposed to NNK and B[a]P. Accordingly, this exposure resulted in induction of the EMT program in epithelial cells, which in turn, supported development of mammospheres and an increase in stem-like cell population. Although cumulative exposures to NNK and B[a]P for 20 cycles failed to induce cellular acquisition of stem-like cell and EMT-associated properties to comparable levels acquired by tumorigenic, malignant MCF10A-Ras cells, the increased degrees of these properties acquired by the non-tumorigenic, pre-malignant NNK- and B[a]P-exposed cells clearly revealed a carcinogenesis progression in an exposure cycle-dependent manner. Cancer stem-like cells have been postulated to play important roles in pre-malignant and malignant stages of cancer development [37-39], and cancer stem-like cells also play an important role in recurrent cancers after chemotherapy [63]. Thus, it is important to consider cellular acquisition of stem-like cell and EMT-associated properties and markers as new targeted endpoints in measuring carcinogenesis progression.

For the first time, we demonstrated the activity of non-cytotoxic dietary green tea and grape seed extracts in suppression of stem-like cell properties and EMT-associated properties and markers induced by long-term exposure to NNK and B[a]P at a bio-achievable dose.

Previously, we showed that GTC and GSPE, at a non-cytotoxic concentration of 40 μg/ml, effectively blocked NNK- and B[a]P-induced acquisition of the cancer-associated properties of reduced dependence on growth factors, anchorage-independent growth, and acinar-conformational disruption [11-15]. Here, we revealed that co-exposure to GTC and GSPE was effective in suppressing cellular acquisition of increased abilities of mammosphere formation, stem-like cell population, cell migration, invasion, and mobility induced by long-term exposure to NNK and B[a]P. The activity of dietary GTC and GSPE to protect epithelial cells from acquiring stem-like cell and EMT-associated properties prevented epithelial cells from producing stem-like and mesenchymal cells. GTC and GSPE suppression of cellular acquisition of stem-like cell- and EMT-associated properties were indicated by reduction of the increased ADLH-positive and $CD44^{+}/CD24^{-}$ cell populations and reversal of changes in expression of E-Cadherin, EpCAM, MMP-9, and Vimentin. However, whether dietary GTC and GSPE are able to protect mammary tissues from acquiring carcinogen-induced stem-like cell and EMT-associated properties to reduce the risk of invasive tumors, indicated by suppression of stem-like cell- and EMT-associated markers, remains to be studied.

Our model presents a unique feature in that it is able to determine chronic breast cell carcinogenesis progression induced by cumulative exposures to carcinogens at a physiologically-achievable dose. Using our model, we demonstrated that chronic carcinogenesis was accompanied with acquisition of stem-like cell and EMT-associated properties and markers. These measurable properties and markers should be considered as new cancer-associated properties in studies of breast cell carcinogenesis and may serve as new targeted endpoints in detection of carcinogenesis progression. Thus, our system provides a platform equipped with measurable targeted endpoints to identify preventive agents effective in suppression of cellular

carcinogenesis induced by long-term exposure to carcinogens. NNK and B[a]P are recognized as potent environmental carcinogens in the development of pulmonary cancers [16,17]. Although NNK and B[a]P may not induce tumorigenic carcinogenesis of breast cells, they induce cellular acquisition of various cancer-associated properties, including stem-like and EMT-associated properties; therefore, their carcinogenic roles in breast cancer development, even in pre-malignant stages, should be recognized. Indeed, prevention of cellular carcinogenesis at various stages is the key to reduce the risk of cancer development, and effective intervention of pre-malignant carcinogenesis is highly important in cancer prevention. It is important to consider the use of non-cytotoxic, dietary GTC and GSPE in early prevention of pre-malignant cell carcinogenesis in sporadic breast cancer development associated with long-term exposure to low doses of environmental carcinogens. Furthering technology of using dietary GTC and GSPE in prevention of pre-malignant cell carcinogenesis, especially to intervene in acquisition of stem-like cell and EMT-associated properties, may allow us to overcome a current obstacle in control of cancer stem-like cell resistance to therapeutic agents.

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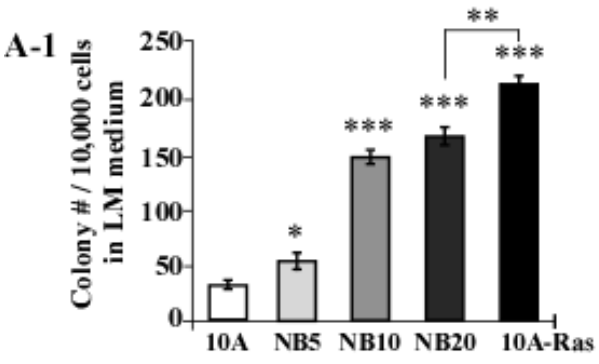
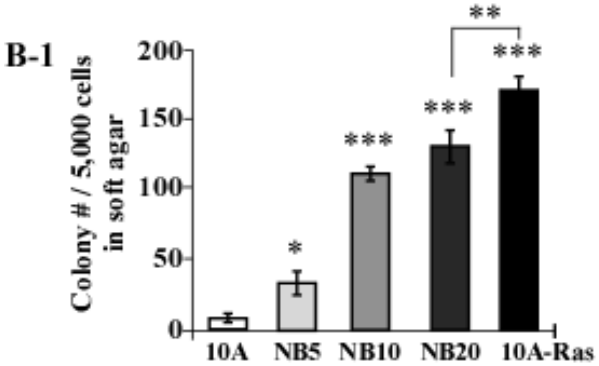
APPENDIX

Figure 4.1. NNK- and B[a]P-induced cellular carcinogenesis. MCF10A (10A) cells were repeatedly exposed to NNK combined with B[a]P each at 100 pmol/L for 5, 10, and 20 cycles, resulting in NB5, NB10, and NB20 cell lines, respectively. MCF10A cells were stably transfected to ectopically express oncogenic H-Ras, resulting in MCF10A-Ras (10A-Ras) cells. (A-1 and A-2) To determine cellular acquisition of the cancer-associated property of reduced dependence on growth factors, cells were seeded in LM medium for 10 d; cell colonies (≥ 0.5 mm diameter) were counted (A-1). (B-1 and B-2) To determine cellular acquisition of the cancer-associated property of anchorage-independent growth, cells were seeded in soft-agar for 20 d; cell colonies (≥ 0.1 mm diameter) were counted (B-1). *Columns*, mean of triplicates; *bars*, SD. All results are representative of 3 independent experiments. The Student *t* test was used to analyze statistical significance, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; α levels were adjusted by the Simes method. (A-2 and B-2) Tables show colony numbers, average colony size, and range of colony size developed in LM medium and soft-agar, respectively. Mean colony numbers in each group were analyzed by one-way ANOVA at $P < 0.001$ to indicate significant difference in number of colonies in various groups. To further determine the significant difference between individual groups, a pairwise analysis of variables was performed using the Duncan multiple range test. Means with different superscript letters (a, b, c, d, and e) indicate significant difference at $P < 0.001$ between groups; no significant difference was seen between groups with the same superscript.



A-2	Colony Number	Colony Size Average, mm	Colony Size Range, mm
10A	41 ± 2^{e}	0.62	0.5-0.8
NB5	63 ± 3^{d}	0.75	0.5-1.0
NB10	154 ± 4^{c}	1.34	0.8-1.4
NB20	168 ± 5^{b}	1.50	1.0-1.7
10A-Ras	209 ± 4^{a}	2.00	1.3-2.2

(Mean ± SD, n=5, P<0.001 one-way ANOVA with pairwise comparison with Duncan's Multiple Range Test. Different subscript letters indicate significant difference.)



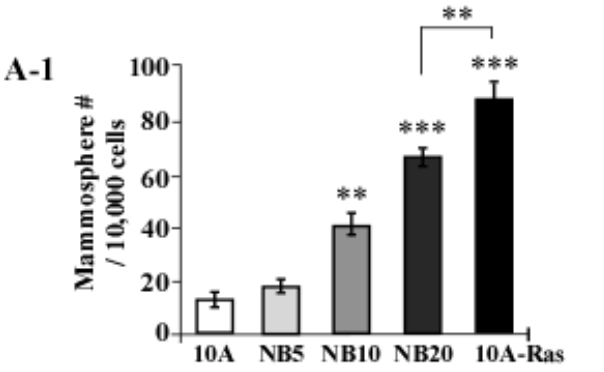
B-2	Colony Number	Colony Size Average, mm	Colony Size Range, mm
10A	4 ± 2^{e}	0.19	0.1-0.2
NB5	23 ± 6^{d}	0.25	0.1-0.3
NB10	120 ± 4^{c}	0.58	0.2-0.9
NB20	138 ± 8^{b}	0.65	0.3-1.0
10A-Ras	168 ± 5^{a}	0.85	0.5-1.1

(Mean ± SD, n=5, P<0.001 one-way ANOVA with pairwise comparison with Duncan's Multiple Range Test. Different subscript letters indicate significant difference.)

Figure 4.1.

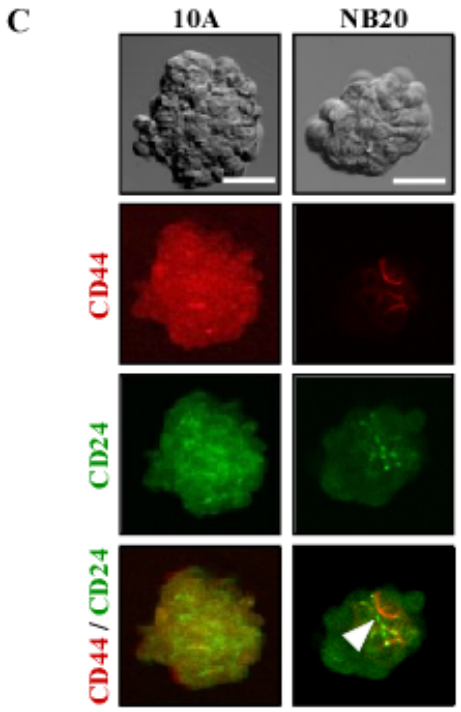
NNK- and B[a]P-induced cellular carcinogenesis.

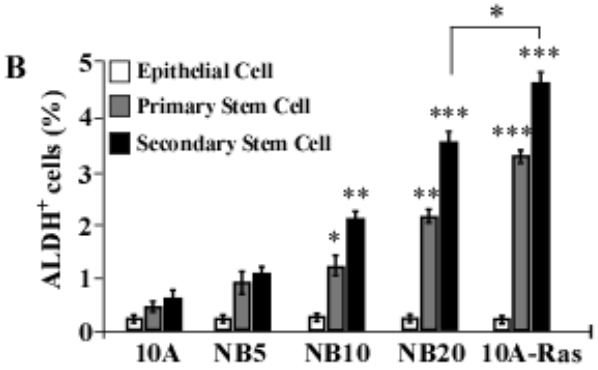
Figure 4.2. NNK- and B[a]P-induced stem-like cell properties. (A-1 and A-2) To determine cellular acquisition of the ability of serum-independent non-adherent growth, 1×10^4 MCF10A (10A), NB5, NB10, NB20, and MCF10A-Ras (10A-Ras) cells were seeded in non-adherent cultures for 10 d; then, mammospheres (≥ 0.1 mm diameter) were counted (A-1). *Columns*, mean of triplicates; *bars*, SD. The Student *t* test was used to analyze statistical significance, indicated by ** $P < 0.01$, *** $P < 0.001$; α levels were adjusted by the Simes method. (A-2) Table shows mammosphere numbers, average size, and range of size. Mean mammosphere numbers in each group were analyzed by one-way ANOVA at $P < 0.001$ to indicate significant difference in number of colonies in various groups. To further determine the significant difference between individual groups, a pairwise analysis of variables was performed using the Duncan multiple range test. Means with different superscript letters (a, b, c, and d) indicate significant difference at $P < 0.001$ between groups; no significant difference was seen between groups with the same superscript. (B) Epithelial (white columns), primary (light grey columns), and secondary (dark grey columns) mammospheres were collected for detection of ALDH-expressing ($ALDH^+$) cell population (%). (C) Representative images of co-immuno-staining of 10A and NB20 mammospheres with PE-conjugated CD44-specific antibody and FITC-conjugated CD24-specific antibody. Bars, 100μm. Arrow head indicates $CD44^+/CD24^-$ cell. (D) Mammospheres were co-labeled with PE-conjugated CD44-specific antibody and FITC-conjugated CD24-specific antibody, and $CD44^+/CD24^-$ cell population (%) was determined by flow cytometry. *Columns*, mean of triplicates; *bars*, SD. All results are representative of at least 3 independent experiments**.** The Student *t* test was used to analyze statistical significance, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; α levels were adjusted by the Simes method.



A-2	Mammosphere Number	Mammosphere Size Average, mm	Range, mm
10A	18 ± 2^{d}	0.15	0.1-0.2
NB5	23 ± 6^{d}	0.15	0.1-0.2
NB10	41 ± 4^{c}	0.20	0.1-0.3
NB20	71 ± 3^{b}	0.25	0.1-0.4
10A-Ras	89 ± 5^{a}	0.28	0.1-0.4

(Mean ± SD, n=5, P<0.001 one-way ANOVA with pairwise comparison with Duncan's Multiple Range Test. Different subscript letters indicate significant difference.)





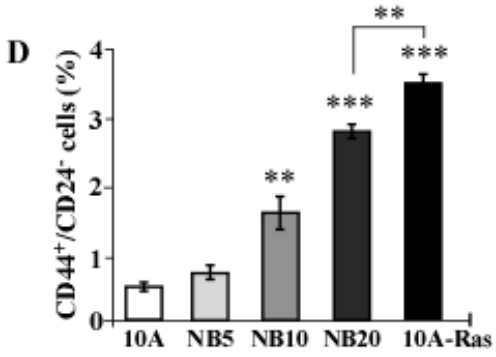


Figure 4.2.

NNK- and B[a]P-induced stem-like cell properties

Figure 4.3. NNK- and B[a]P-induced EMT-associated properties. (A) Representative morphological features of MCF10A (10A), NB10, NB20, and MCF10A-Ras (10A-Ras) cells, 400X. (B) Cell lysates were analyzed by Western immunoblotting using specific antibodies to detect levels of EpCAM, E-Cadherin, MMP-9, and Vimentin, with β-Actin as a control, and these levels were quantified by densitometry. The levels of EpCAM, E-Cadherin, MMP-9, and Vimentin were calculated by normalizing with the level of β-Actin and the level set in 10A cells (lane 1) as 1 (X, arbitrary unit). All results are representative of at least 3 independent experiments. (C) Cellular migratory and (D) invasive activities were determined by counting the numbers of cells translocated through polycarbonate filter without or with coated Matrigel, respectively, in 10 arbitrary visual fields. *Columns*, mean of triplicates; *bars*, SD. The Student *t* test was used to analyze statistical significance, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; α levels were adjusted by the Simes method. (E-1 and E-2) Cellular acquisition of increased mobility was determined by wound healing assay. Cells were seeded in CM medium and grown to confluence (E-1, a); then, a linear area of cell layer was removed from each culture with a 23-gauge needle to produce wounded cultures, and the wounded areas were examined (×100 magnification) 6 (b) and 20 h (c) after wounding. Arrows indicate width of wounded areas. Results are representative of 3 independent experiments. (E-2) To quantitatively measure cell mobility detected in (E-1), the area not healed by the cells was subtracted from the total area of the initial wound to calculate the wound healing area (%) at time intervals of 6 (white columns) and 20 h (grey columns). *Columns*, mean of triplicates; *bars*, SD. All results are representative of 3 independent experiments. The Student *t* test was used to analyze statistical significance, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; α levels were adjusted by the Simes method.

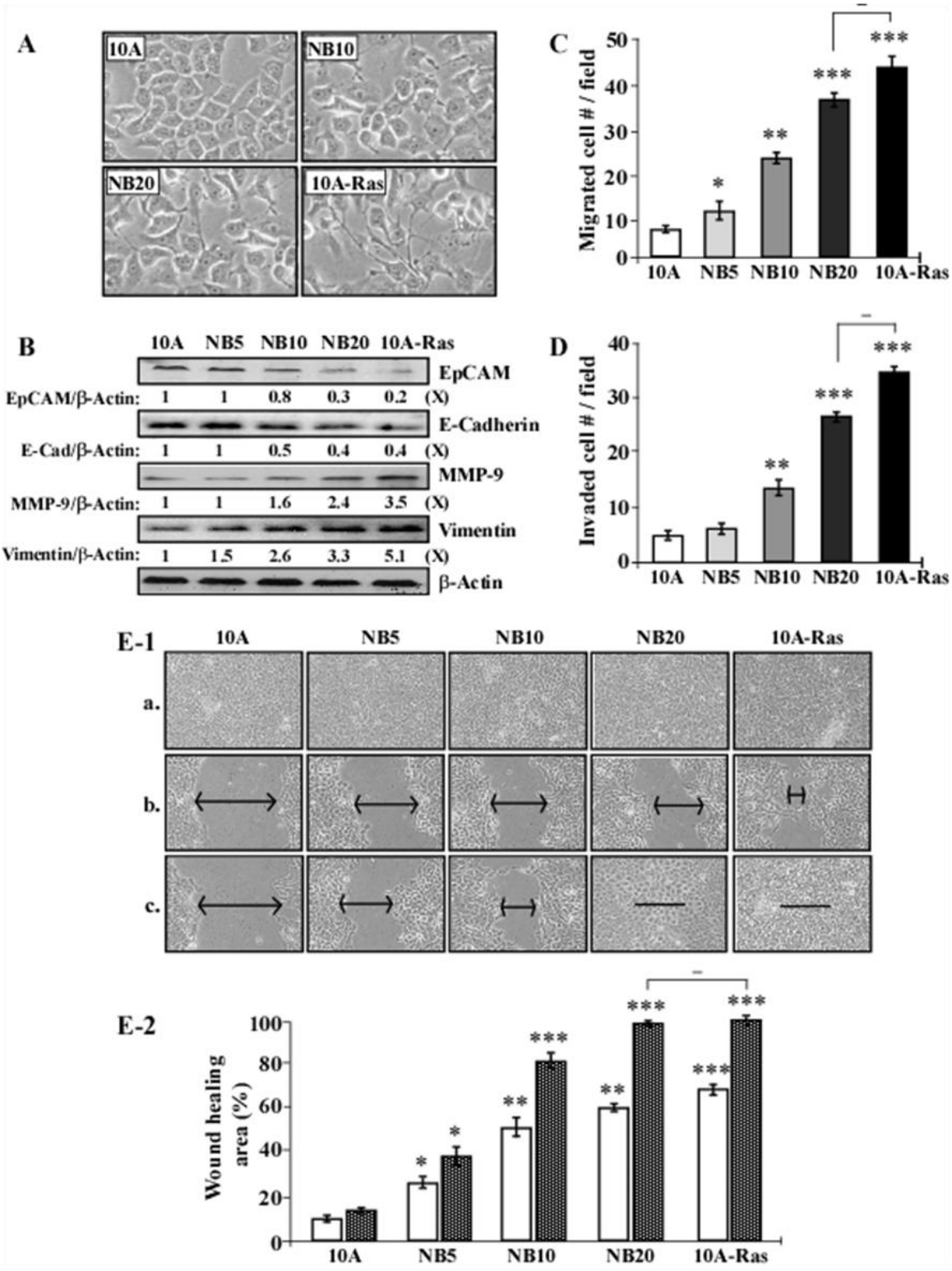
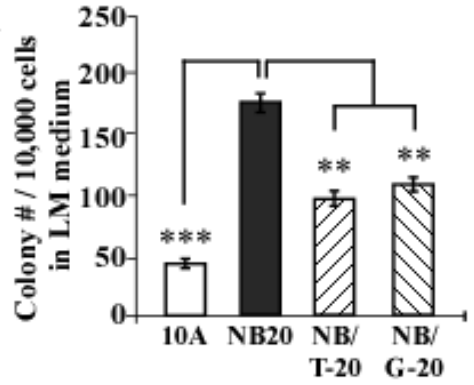


Figure 4.3.

NNK- and B[a]P-induced EMT-associated properties.

Figure 4.4. GTC and GSPE suppression of NNK- and B[a]P-induced cellular carcinogenesis. MCF10A (10A) cells were repeatedly exposed to NNK combined with B[a]P each at 100 pmol/L in the presence of GTC and GSPE at 40μg/ml for 20 cycles, resulting in NB/T-20 and NB/G-20 cell lines, respectively. (A-1 and A-2) To determine acquisition of the cancer-associated property of reduced dependence on growth factors, 10A, NB20, NB/T-20, and NB/G-20 cells were seeded in LM medium for 10 d; cell colonies (≥ 0.5 mm diameter) were counted (A-1). (B-1 and B-2) To determine cellular acquisition of the cancer-associated property of anchorage-independent growth, cells were seeded in soft agar for 20 d; cell colonies (≥ 0.1 mm diameter) were counted (B-1). *Columns*, mean of triplicates; *bars*, SD. All results are representative of 3 independent experiments. The Student *t* test was used to analyze statistical significance, indicated by ** $P < 0.01$, *** $P < 0.001$; α levels were adjusted by the Simes method. (A-2 and B-2) Tables show colony numbers, average colony size, and range of colony size developed in LM medium and soft agar, respectively. Mean colony numbers in each group were analyzed by one-way ANOVA at $P < 0.001$ to indicate significant difference in number of colonies in various groups. To further determine the significant difference between individual groups, a pairwise analysis of variables was performed using the Duncan multiple range test. Means with different superscript letters (a, b, and c) indicate significant difference at $P < 0.001$ between groups; no significant difference was seen between groups with the same superscript.

A-1

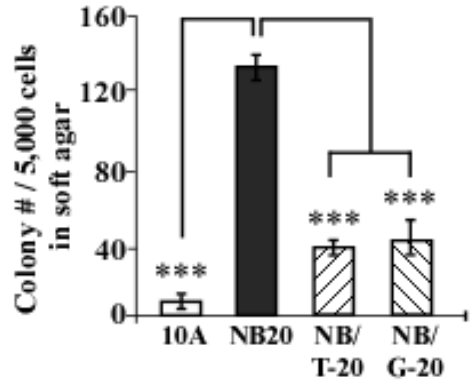


A-2

	Colony Number	Colony Size Average, mm	Range, mm
10A	41 ± 2^{c}	0.62	0.5-0.8
NB20	168 ± 3^{a}	1.50	1.0-1.7
NB/T-20	96 ± 4^{b}	0.90	0.8-1.0
NB/G-20	104 ± 5^{b}	0.95	0.8-1.1

(Mean ± SD, n=5, P<0.001 one-way ANOVA with pairwise comparison with Duncan's Multiple Range Test. Different subscript letters indicate significant difference.)

B-1



B-2

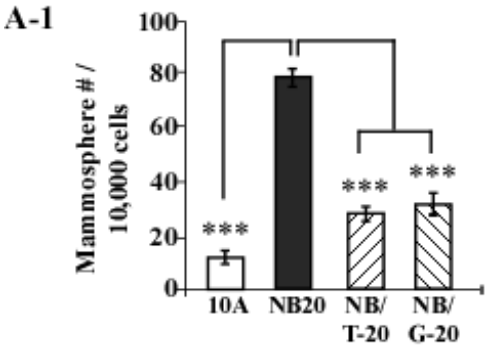
	Colony Number	Colony Size Average, mm	Range, mm
10A	4 ± 2^{c}	0.19	0.1-0.2
NB20	138 ± 6^{a}	0.65	0.3-1.0
NB/T-20	41 ± 4^{b}	0.34	0.2-0.4
NB/G-20	45 ± 8^{b}	0.38	0.2-0.4

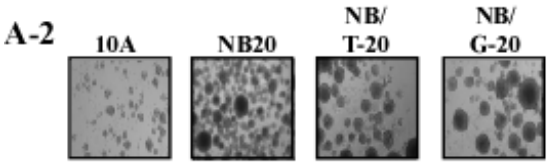
(Mean ± SD, n=5, P<0.001 one-way ANOVA with pairwise comparison with Duncan's Multiple Range Test. Different subscript letters indicate significant difference.)

Figure 4.4.

GTC and GSPE suppression of NNK- and B[a]P-induced cellula carcinogenesis.

Figure 4.5. GTC and GSPE suppression of NNK- and B[a]P-induced stem-like cell properties. (A-1 to A-3) To determine cellular acquisition of the ability of serum-independent non-adherent growth, 1×10^4 MCF10A (10A), NB20, NB/T-20, and NB/G-20 cells were seeded in non-adherent cultures for 10 d; then, mammospheres ($\geq$ 0.1 mm diameter) were counted (A-1). *Columns*, mean of triplicates; *bars*, SD. The Student *t* test was used to analyze statistical significance, indicated by *** $P < 0.001$; α levels were adjusted by the Simes method. (A-2) Representative fields of mammosphere cultures. (A-3) Table shows mammosphere numbers, average size, and range of size. Mean mammosphere numbers in each group were analyzed by one-way ANOVA at $P < 0.001$ to indicate significant difference in number of colonies in various groups. To further determine the significant difference between individual groups, a pairwise analysis of variables was performed using the Duncan multiple range test. Means with different superscript letters (a, b, and c) indicate significant difference at $P < 0.001$ between groups; no significant difference was seen between groups with the same superscript. (B) Parental epithelial cells (white columns) and the primary (grey columns) and secondary (dark columns) mammospheres were trypsinized, and ALDH-expressing ($ALDH^+$) cell population (%) was determined by flow cytometry. (C) Mammospheres were trypsinized, cells were incubated with PE-conjugated CD44-specific antibody and FITC-conjugated CD24-specific antibody, and $CD44^+/CD24^-$ cell population (%) was determined by flow cytometry. *Columns*, mean of triplicates; *bars*, SD. All results are representative of at least 3 independent experiments. The Student *t* test was used to analyze statistical significance, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; α levels were adjusted by the Simes method.

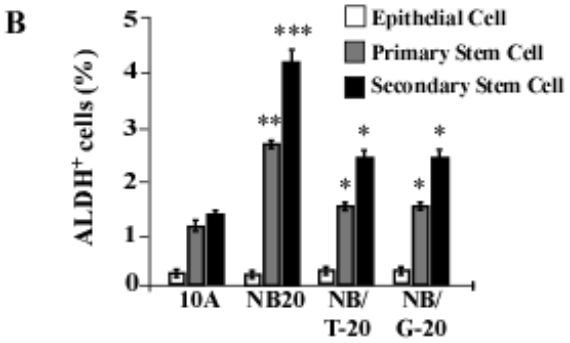




A-3

	Mammosphere Number	Mammosphere Size Average, mm	Range, mm
10A	18 ± 2 ^{c}	0.15	0.1-0.2
NB20	71 ± 3 ^{a}	0.25	0.1-0.4
NB/T-20	30 ± 4 ^{b}	0.14	0.1-0.3
NB/G-20	35 ± 6 ^{b}	0.18	0.1-0.3

(Mean $\pm$ SD, n=5, P<0.001 one-way ANOVA with pairwise comparison with Duncan's Multiple Range Test. Different subscript letters indicate significant difference.)



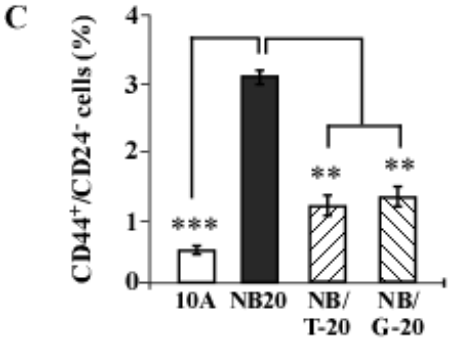


Figure 4.5.

GTC and GSPE suppression of NNK- and B[a]P-induced stem-like cell properties.

Figure 4.6. GTC and GSPE suppression of NNK- and B[a]P-induced EMT-associated properties. (A) Representative morphological features of MCF10A (10A), NB20, NB/T-20, and NB/G-20 cells, 400X. (B) Cell lysates were analyzed by Western immunoblotting using specific antibodies to detect levels of EpCAM, E-Cadherin, MMP-9, and Vimentin, with β-Actin as a control, and these levels were quantified by densitometry. The levels of EpCAM, E-Cadherin, MMP-9, and Vimentin were calculated by normalizing with the level of β-Actin and the level set in 10A cells (lane 1) as 1 (X, arbitrary unit). (C) Cellular migratory and (D) invasive activities were determined by counting the numbers of cells translocated through polycarbonate filter without or with coated Matrigel, respectively, in 10 arbitrary visual fields. *Columns*, mean of triplicates; *bars*, SD. The Student *t* test was used to analyze statistical significance, indicated by ** $P < 0.01$, *** $P < 0.001$; α levels were adjusted by the Simes method. (E-1 and E-2) Cellular acquisition of increased mobility was determined by wound healing assay. Cells were seeded in CM medium and grown to confluence (E-1, a); then, a linear area of cell layer was removed from each culture with a 23-gauge needle to produce wounded cultures, and the wounded areas were examined (×100 magnification) 6 (b) and 20 h (c) after wounding. Arrows indicate width of wounded areas. Results are representative of 3 independent experiments. (E-2) To quantitatively measure cell mobility detected in (E-1), the area not healed by the cells was subtracted from the total area of the initial wound to calculate the wound healing area (%) at time intervals of 6 (white columns) and 20 h (grey columns). *Columns*, mean of triplicates; *bars*, SD. The Student *t* test was used to analyze statistical significance, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; α levels were adjusted by the Simes method.

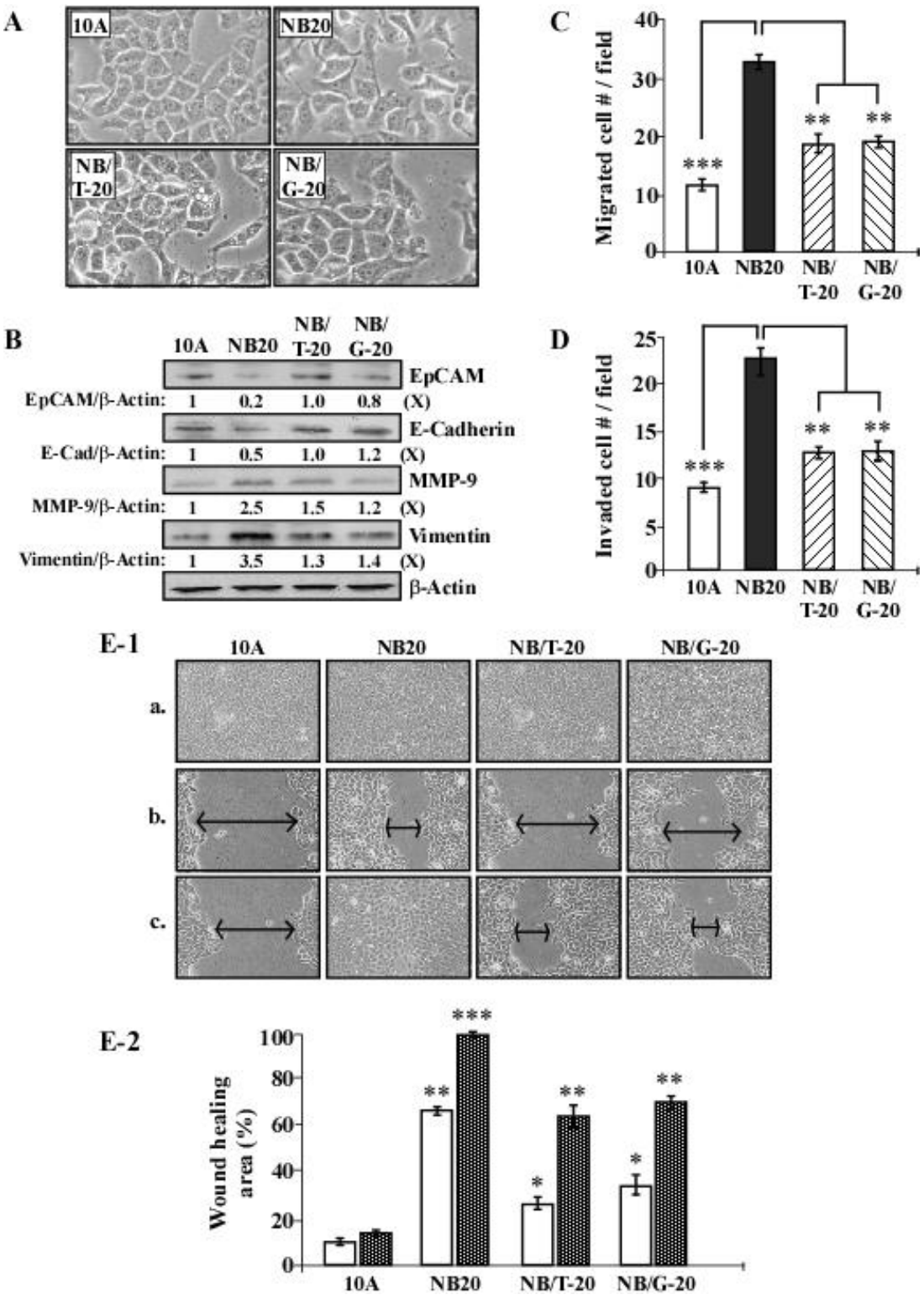


Figure 4.6.

GTC and GSPE suppression of NNK- and B[a]P-induced EMT-associated properties

PART-V

General Discussion

General Discussion

The study presented in the dissertation was designed to (1) investigate the intervention of chronic breast cell carcinogenesis induced by environmental carcinogens in MCF10A cells by dietary compounds like green tea catechins and grape seed extract and, (2) study the importance of epithelial to mesenchymal transition and stem-like cells in chronically induced carcinogenesis.

Intervention of chronic breast cell carcinogenesis induced by environmental carcinogens in MCF10A cells

Prevention of human breast cell carcinogenesis associated with chronic exposure to low doses of environmental carcinogens is an under-investigated area. Our model system presents unique features of mimicking chronically-induced carcinogenesis of human breast cells to increasingly acquire cancer-associated properties induced by chronic, cumulative exposures to carcinogens at low concentrations in the pico-molar range, as in environmental exposure. In contrast, many cell systems have been developed to study the activity of carcinogens at high concentrations in the micro-molar range, as in occupational exposure, in acute induction of cellular carcinogenesis [1-8]. Using our cellular model as a target system, we are able to verify the preventive activity of complete green tea extract and individual catechins (at non-cytotoxic levels) in suppression of chronic cellular carcinogenesis. In addition we have identified the mechanisms through which the catechins can counteract the biological, biochemical, and molecular effects of NNK and B[a]P. Based on our results, use of non-cytotoxic catechin components should be seriously considered in prevention of cellular carcinogenesis induced by chronic exposure to environmental carcinogens.

Our studies revealed that cumulative exposures to NNK and/or B[a]P at a bio-achievable concentration of 100 pmol/L resulted in progression of human breast cells to increasingly acquire cancer-associated properties in an exposure-dependent manner, without acquiring tumorigenicity [9-12]. Although cellular acquisition of tumorigenicity is regarded as the gold standard for validating cell malignancy, many human cancer cells, such as MDA-MB-453 [13] and urinary bladder cancer J82 cells are not tumorigenic [14]. We have shown that cumulative exposures of MCF10A cells to NNK and B[a]P at 100 pmol/L for 10 and 20 cycles induce the cancer-associated property of reduced dependence on growth factors, anchorage independent growth , increased cell motility and acinar-conformational disruption with irregular spheroids developed on Matrigel [9-12]. Green tea extract and individual catechins were able to suppress these carcinogen induced cancerous properties. Acinar structures with a hollow lumen and apicobasally polarized cells are important characteristics found in glandular epithelia in vivo. The disruption of an intact glandular structure is a hallmark of epithelial cancer, even at its earliest premalignant stages, such as ductal carcinoma *in-situ* (DCIS) [15-17]. Clinically, breast cells involved in DCIS are not malignant and have not acquired the ability to invade adjacent tissues through the ductal or lobular wall, but often premalignant cells can either develop into malignant cells or increase the risk of becoming malignant [3,18,19]. Thus, in addition to tumorigenicity, using various cancer-associated properties as measurable targeted endpoints should be seriously considered in studying cellular carcinogenesis and intervention of cellular carcinogenesis.

Short-term exposure to NNK and B[a]P at 100 pmol/L induced transient ROS elevation leading to ERK pathway activation, cell proliferation, and chromosomal DNA damage. However, these short-term targeted endpoints were transient; they were not permanent in cells

acquiring cancer-associated properties induced by cumulative exposures to NNK and B[a]P. Aiming at these transiently-induced, short-term targeted endpoints, we observed that green tea catechins EC, ECG, EGC, and EGCG, at a non-cytotoxic concentration of 10 μg/mL, were capable of blocking NNK- and B[a]P-induced ROS elevation, ERK pathway activation, cell proliferation, and DNA damage to various extents in non-cancerous MCF10A and in adenocarcinoma MCF7 cells. Interestingly, ECG was more effective than EC, EGC, and EGCG in suppression of cellular carcinogenesis, measured by degrees of acquired cancer-associated properties in long-term targeted carcinogenic endpoints, permanently induced by cumulative exposures to NNK and B[a]P. However, whether the activity of individual catechins may vary in suppression of cellular carcinogenesis induced by other carcinogens remains to be addressed. ECG was also less cytotoxic to MCF10A cells than EGCG and EGC. Studies of the hepatotoxicity of GTCs in rats showed that EGCG is the most toxic of all four catechins; for example, the LD_{50} for EGCG (200 μM) was 10 times lower than that for ECG (2000 μM), indicating that ECG is much less toxic than EGCG [20]. Thus, the cytotoxicity-independent ability of these catechins to block NNK- and B[a]P-induced, ROS elevation, ERK pathway activation, cell proliferation, and DNA damage in each short-term exposure accounted for their effectiveness in suppression of breast cell carcinogenesis induced by long-term, accumulated exposure to NNK and B[a]P.

NNK- and B[a]P-induced ROS elevation played a key role in activation of the ERK pathway, leading to cell proliferation and DNA damage. Studies have shown that ROS is able to induce the ERK pathway via activation of membrane-associated growth factor receptors or via Raf-independent Mek1/2 activation [21,22]. In our studies, exposure of MCF10A cells to NNK and B[a]P did not induce any detectable upregulation of Ras or Raf, both of which are upstream

from Mek1/2 and Erk1/2. Thus, NNK- and B[a]P-elevated ROS induced the ERK pathway in a Raf-independent manner. However, how ROS was induced by NNK and B[a]P in MCF10A and MCF7 cells, and how ROS was able to induce Raf-independent activation of Mek1/2 and Erk1/2 remain to be determined.

Our model system addresses breast cell carcinogenesis induced by chronic exposure to carcinogens at bio-achievable levels and identifies preventive agents, at non-cytotoxic levels, capable of suppressing chronically-induced breast cell carcinogenesis. We demonstrated, for the first time, use of a new powerful two-step strategy. The first step uses short-term biological, biochemical, and molecular targeted carcinogenic endpoints transiently induced by short-term exposure to carcinogens for detecting preventive agents capable of blocking cellular carcinogenesis. The second step subsequently uses long-term biological, biochemical, and molecular targeted carcinogenic endpoints induced by chronic exposure to carcinogens to verify preventive agents effective in suppression of cellular carcinogenesis. Our model system presents unique features of mimicking chronically-induced carcinogenesis of human breast cells to increasingly acquire cancer-associated properties induced by chronic, cumulative exposures to carcinogens at low concentrations in the pico-molar range, as in environmental exposure. Using our model system will conceivably accelerate the identification of additional preventive agents that are effective in reducing the health risk of sporadic breast cancer associated with chronic exposure to carcinogens present in environmental pollution.

The importance of epithelial to mesenchymal transition and stem-like cells in chronically induced carcinogenesis and its dietary prevention

In this study, we presented results that reveal the value of acquisition of stem-like cell properties as well as EMT-associated markers and properties, in measuring the induction of chronic cellular carcinogenesis. Epithelial cells chronically exposed to NNK and B[a]P increasingly acquired stem-like cell properties of mammosphere formation and increased populations of ADLH-positive and $CD44^{+}/CD24^{-}$ stem-like cells in mammospheres. The ability of carcinogen-exposed epithelial cells to develop mammospheres in serum-free non-adherent cultures may be related to their combined abilities of reduced dependence on growth factors and anchorage-independent growth. The ability of carcinogenic epithelial cells to produce increased stem-like cell populations in mammospheres may be mediated by the EMT program, as indicated by many previous studies [23-25]. Our results revealed that the EMT-associated properties of mesenchymal morphology, cell migration, invasion, and mobility, as well as the EMT-associated markers of losing E-Cadherin and EpCAM and gaining MMP-9 and Vimentin were also increasingly acquired by epithelial cells chronically exposed to NNK and B[a]P. Accordingly, this exposure resulted in induction of the EMT program in epithelial cells, which in turn, supported development of mammospheres and an increase in stem-like cell population. Although cumulative exposures to NNK and B[a]P for 20 cycles failed to induce cellular acquisition of stem-like cell and EMT-associated properties to comparable levels acquired by tumorigenic, malignant MCF10A-Ras cells, the increased degrees of these properties acquired by the non-tumorigenic, pre-malignant NNK- and B[a]P-exposed cells clearly revealed a carcinogenesis progression in an exposure cycle-dependent manner. Cancer stem-like cells have been postulated to play important roles in pre-malignant and malignant stages of cancer development [26-28],

and cancer stem-like cells also play an important role in recurrent cancers after chemotherapy [29]. Thus, it is important to consider cellular acquisition of stem-like cell and EMT-associated properties and markers as new targeted endpoints in measuring carcinogenesis progression.

For the first time, we demonstrated the activity of non-cytotoxic dietary green tea and grape seed extracts in suppression of stem-like cell properties and EMT-associated properties and markers induced by long-term exposure to NNK and B[a]P at a bio-achievable dose. Previously, we showed that GTC and GSPE, at a non-cytotoxic concentration of 40 μg/ml, effectively blocked NNK- and B[a]P-induced acquisition of the cancer-associated properties of reduced dependence on growth factors, anchorage-independent growth, and acinar-conformational disruption [9-12]. Here, we revealed that co-exposure to GTC and GSPE was effective in suppressing cellular acquisition of increased abilities of mammosphere formation, stem-like cell population, cell migration, invasion, and mobility induced by long-term exposure to NNK and B[a]P. The activity of dietary GTC and GSPE to protect epithelial cells from acquiring stem-like cell and EMT-associated properties prevented epithelial cells from producing stem-like and mesenchymal cells. GTC and GSPE suppression of cellular acquisition of stem-like cell- and EMT-associated properties were indicated by reduction of the increased ADLH-positive and $CD44^{+}/CD24^{-}$ cell populations and reversal of changes in expression of E-Cadherin, EpCAM, MMP-9, and Vimentin. However, whether dietary GTC and GSPE are able to protect mammary tissues from acquiring carcinogen-induced stem-like cell and EMT-associated properties to reduce the risk of invasive tumors, indicated by suppression of stem-like cell- and EMT-associated markers, remains to be studied.

Our model presents a unique feature in that it is able to determine chronic breast cell carcinogenesis progression induced by cumulative exposures to carcinogens at a physiologically-

achievable dose. Using our model, we demonstrated that chronic carcinogenesis was accompanied with acquisition of stem-like cell and EMT-associated properties and markers. These measurable properties and markers should be considered as new cancer-associated properties in studies of breast cell carcinogenesis and may serve as new targeted endpoints in detection of carcinogenesis progression. Thus, our system provides a platform equipped with measurable targeted endpoints to identify preventive agents effective in suppression of cellular carcinogenesis induced by long-term exposure to carcinogens. NNK and B[a]P are recognized as potent environmental carcinogens in the development of pulmonary cancers [30,31]. Although NNK and B[a]P may not induce tumorigenic carcinogenesis of breast cells, they induce cellular acquisition of various cancer-associated properties, including stem-like cell and EMT-associated properties; therefore, their carcinogenic roles in breast cancer development, even in pre-malignant stages, should be recognized. Indeed, prevention of cellular carcinogenesis at various stages is the key to reduce the risk of cancer development, and effective intervention of pre-malignant carcinogenesis is highly important in cancer prevention. It is important to consider the use of non-cytotoxic, dietary GTC and GSPE in early prevention of pre-malignant cell carcinogenesis in sporadic breast cancer development associated with long-term exposure to low doses of environmental carcinogens. Furthering technology of using dietary GTC and GSPE in prevention of pre-malignant cell carcinogenesis, especially to intervene in acquisition of stem-like cell and EMT-associated properties, may allow us to overcome a current obstacle in control of cancer stem-like cell resistance to therapeutic agents.

Summary

This study has shown that repeated exposure of non-cancerous, human breast epithelial MCF10A cells with low dose environmental carcinogens NNK and B[a]P induces various cancer-associated properties like reduced dependence on growth factors, anchorage independent growth, increased cell motility and acinar-conformational disruption. We detected the ability of dietary compounds like green tea extract, individual catechins from green tea and grape seed, at non-cytotoxic concentrations to suppress chronically -induced carcinogenesis of breast epithelial cells. This is summarized in the scheme shown in **Figure 5.1**.

To identify the mechanism involved in chronic carcinogenesis we identified short-term biological, biochemical, and molecular targeted carcinogenic endpoints transiently induced by short-term exposure to carcinogens for detecting preventive agents capable of blocking cellular carcinogenesis. Short-term exposure to NNK and B[a]P at 100 pmol/L induced transient ROS elevation leading to ERK pathway activation, cell proliferation, and chromosomal DNA damage (**Figure 5.2**). We also observed that green tea catechins EC, ECG, EGC, and EGCG, at a non-cytotoxic concentration of 10 µg/mL, were capable of blocking NNK- and B[a]P-induced ROS elevation, ERK pathway activation, cell proliferation, and DNA damage to various extents.

We further investigated stem-like cell- and EMT-associated properties and markers progressively induced by chronic exposure of breast epithelial cells to NNK and B[a]P. We also used stem-like cell- and EMT-associated properties and markers as targeted endpoints to verify the activity of non-cytotoxic GTC and GSPE in suppression of NNK- and B[a]P-induced cellular carcinogenesis.

Prospects

Our study shows that NNK- and B[a]P-elevated ROS induced the ERK pathway in a Raf-independent manner. However, how ROS was induced by NNK and B[a]P in MCF10A and MCF7 cells, and how ROS was able to induce Raf-independent activation of Mek1/2 and Erk1/2 remain to be determined.

We have shown the exposure of MCF10A cells to NNK and B[a]P at 100 pmol/L for 20 cycles can induce various cancer-associated properties, but no tumorigenicity in mice, therefore it is important to identify that if further treatment with carcinogens will render the cell to be tumorigenic or whether treating the with additional carcinogens might lead to tumorigenicity.

GTC and GSPE suppression of cellular acquisition of stem-like cell- and EMT-associated properties were indicated by reduction of the increased ADLH-positive and $CD44^{+}/CD24^{-}$ cell populations and reversal of changes in expression of E-Cadherin, EpCAM, MMP-9, and Vimentin. However, whether or not dietary GTC and GSPE are able to protect mammary tissues from acquiring carcinogen-induced stem-like cell and EMT-associated properties to reduce the risk of invasive tumors, indicated by suppression of stem-like cell- and EMT-associated markers, remains to be studied.

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APPENDIX

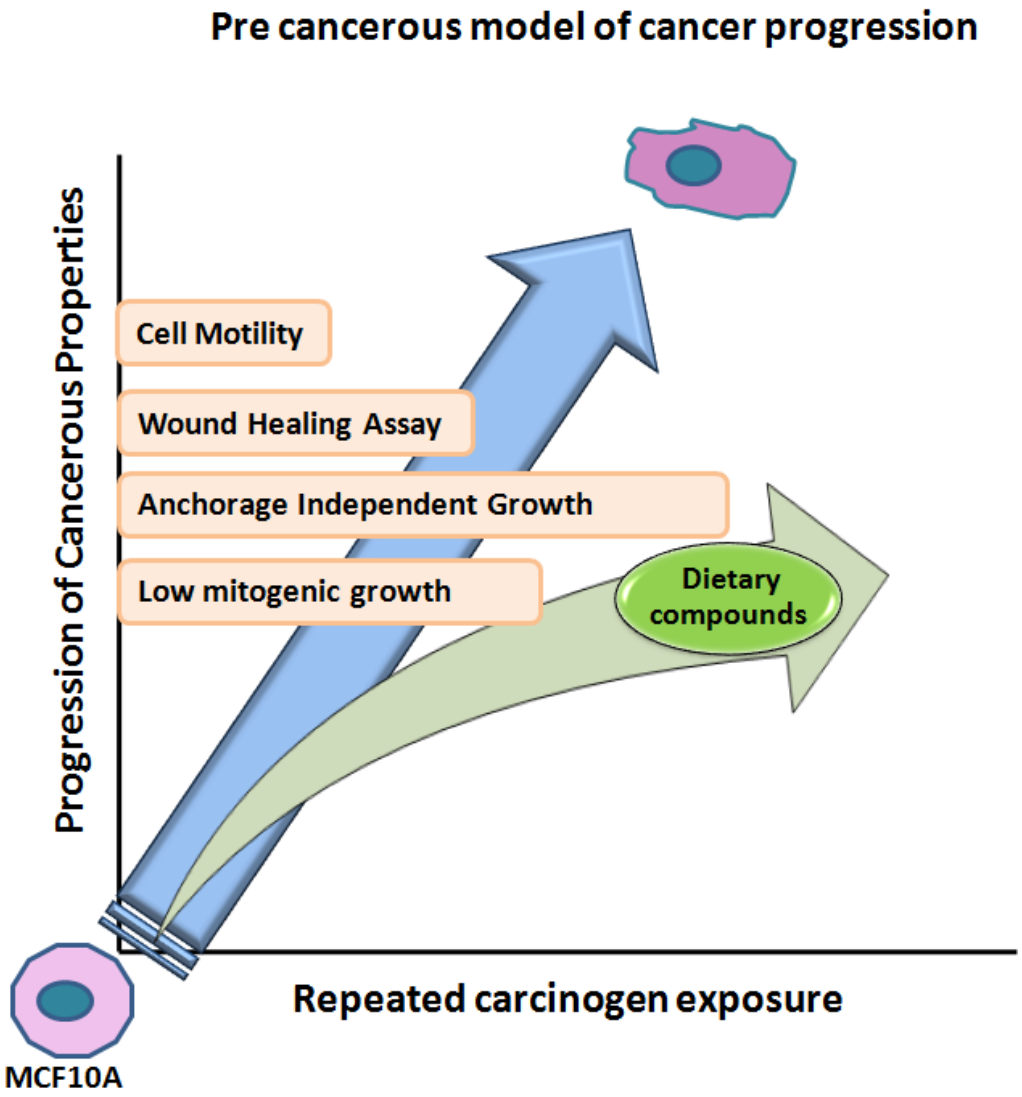

Figure 5.1

Schematic indication rogressive induction of cancer-associated properties with exposure to carcinogens and its suppression by dietary compounds.

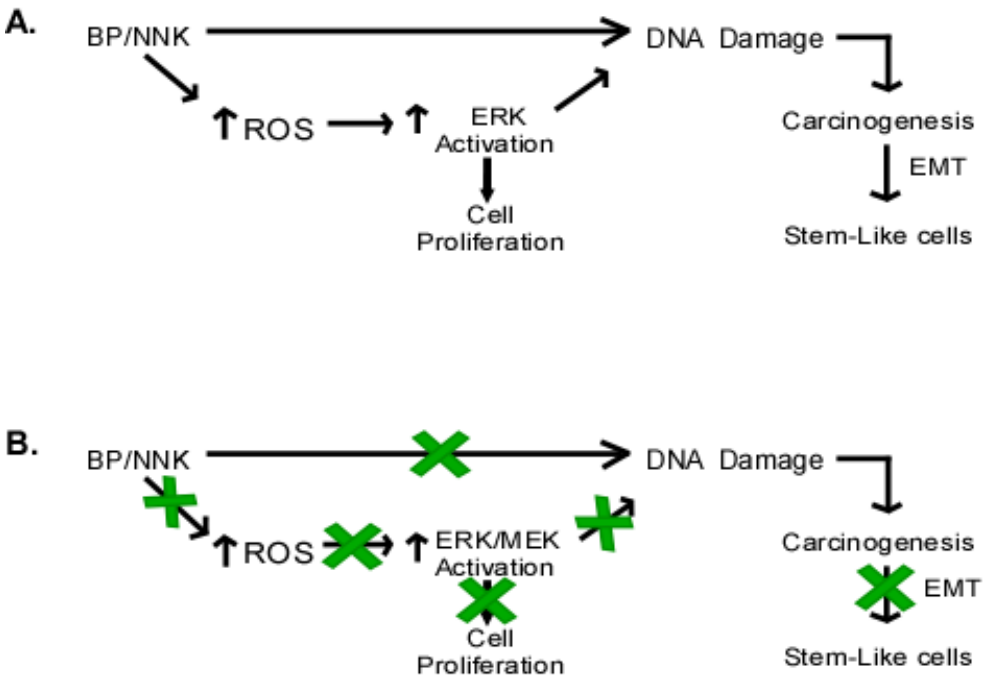

Figure 5.2

(A) Hypothetical scheme of carcinogen-induced ROS, cell proliferation and DNA damage

(B) Hypothetical scheme of suppression of carcinogen-induced ROS, cell proliferation and DNA damage by green tea (indicated by green cross).

VITA

Kusum Rathore was born in Jaipur, Rajasthan, India on the 10th of September, 1983. She completed her primary and advanced level education in Saint Angela Sophia School, Jaipur. She earned her B.S. in Industrial Microbiology at The Maharani College, University of Rajasthan, in July, 2005. She pursued her M.S. in Biomedical Sciences at Ambedkar Centre for Biomedical Research, The University of Delhi, India in July 2007. She joined The University of Tennessee, Knoxville in August 2007 USA, and received a Doctor of Philosophy in Life Science, at The School of Genome Science and Technology, University of Tennessee in December 2011. She plans to pursue her post doctoral training.