

## A Mechanism-Based *In Vitro* Anticancer Drug Screening Approach for Phenolic Phytochemicals

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**Abstract:** Plant-derived phenolic compounds, including polyphenols (*e.g.*, tannins), flavonoids, and phenolic acids, have been under investigation for their anticancer therapeutic and chemoprevention properties. Recently, certain mechanisms underlying the differential effects of GTPPs on tumor versus normal cells have been determined. These suggest that GTPPs may simultaneously activate multiple pathways. However, existing screening methods are insufficient for the identification of agents that possess both a cytotoxic effect on tumor cells and a protective effect on normal cells. The current study describes the establishment of an *in vitro* survival/apoptosis testing system based on detecting these mechanisms by a double-fluorescence method. This system is able to screen potential chemopreventive or therapeutic agents from (but not limited to) plant-derived compounds based on the pathways differentially activated by the agents. Tumor cell death and normal cell survival are detected simultaneously, in a device that co-cultures normal human cells adjacent to human tumor cells.

### Introduction

**A**N IDEAL ANTICANCER DRUG would simultaneously destroy tumor cells efficiently, but protect adjacent normal cells. Recently, attention has been given to the chemopreventive and therapeutic effects of naturally occurring phenolic phytochemicals due to their anticancer potential. It is becoming apparent from tests in cell cultures, animal models, and human trials that certain plant-derived phenolic compounds, such as GTPPs (catechins) found in the tea plant (*Camellia sinensis*), are able to induce the desirable differential effect of killing cancer cells and promoting normal cell survival.<sup>1-7</sup> Thus, these compounds have a strong potential for clinical applications. We hypothesized that GTPPs are able to activate

multiple pathways, depending on the cell type. Normal human epithelial cells have been shown to respond to GTPPs by activating a survival pathway associated with modulation of cell differentiation and proliferation, specifically with induction of p57/KIP2, a cell cycle and differentiation regulator. Induction of p57 by GTPPs in normal epithelial cells is necessary and sufficient for cell survival.<sup>5-7</sup> In contrast, when exposed to GTPPs, tumor cells lack a p57 response. This results in a caspase 3-dependent apoptosis. GTPPs, either as a mixture or as the most abundant GTPP, EGCG, induce apoptosis in many types of tumor cells.<sup>3</sup> Pathology studies demonstrated that tumor specimens express lower levels of p57 protein compared with paired normal tissues, and low levels of p57 often correlate with poor prognosis.<sup>8-11</sup> The differ-

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**ABBREVIATIONS:** BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EGCG, (-)-epigallocatechin-3-gallate; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GTPP, green tea polyphenol; KGM-2, keratinocyte growth medium-2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NHEK, normal human primary epidermal keratinocytes; OSC-GFP, green fluorescent protein-expressing OSC-2 cell line; PBS, phosphate-buffered saline.

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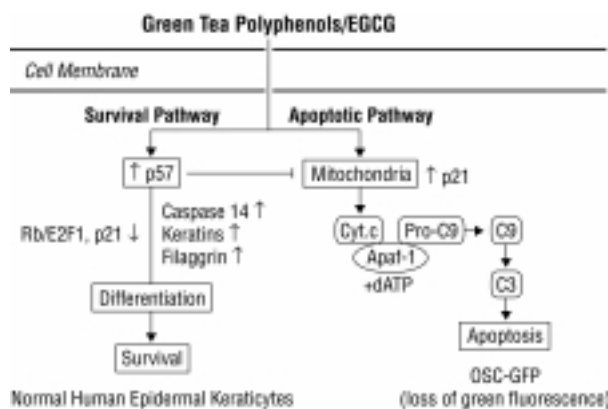
ential effect of GTPPs/EGCG and signal pathways are summarized in Fig. 1.

We previously reported that GTPP-induced apoptosis occurred in various oral carcinoma and breast cancer cells.<sup>12</sup> The GTPP-induced apoptosis is mitochondria-mediated and caspase 3-dependent, as confirmed by caspase 3 activity assay, annexin V apoptosis assay, and the MTT assay. Importantly, caspase 3-deficient cells are resistant to GTPP-induced apoptosis, but become sensitive after stable transfection with wild-type caspase 3.<sup>6,7,12</sup> The oral carcinoma cell line OSC-2 showed high sensitivity to GTPPs.<sup>6,12–14</sup> OSC-2 cells stably transfected with GFP cDNA, OSC-GFP, maintained the high sensitivity to GTPPs as measured by caspase 3 activity and MTT assays (unpublished observations). Importantly, the green fluorescence diminished when OSC-GFP cells were induced to apoptosis by GTPPs or EGCG.<sup>7</sup> Thus, both OSC-2 and OSC-GFP lines have been used for detecting activation of apoptosis by GTPPs, including the polyphenol EGCG. These observations suggest that following exposure to plant-derived phenolics, p57 induction can be used as a marker for cell survival in human epithelial cells, and the activation of the apoptotic pathway (detected by diminished green fluorescence in OSC-GFP cells) can be used as a marker for tumor cell destruction (Fig. 1). Here we demonstrate a proof-of-principle for an

*in vitro* co-culture system for anticancer drug screening based on double fluorescent detection of these two pathways activated by plant-derived phenolic compounds. However, this system may also be used to test the potency/efficacy of potential or currently available medications or products that possess chemopreventive or therapeutic properties. The unique figure of our system is the ability to detect tumor cell death and normal cell survival in a device in which normal human epithelial cells are co-cultured with human tumor cells. Although several *in vitro* co-culture systems using paired normal and malignant cells that mimic the *in vivo* environment have been developed for anticancer drug screening,<sup>15–17</sup> these systems were not based on intracellular activation of specific pathways, and are not able to mimic human epidermal or mucosal tissues.

The advantages of using a co-culture screening system include the following: (a) it more closely resembles the *in vivo* environment where normal cells and tumor cells are adjacent and interacting; (b) it reduces variation caused by separate culture of normal and tumor cells; (c) it facilitates elimination of a “false positive” agent, *e.g.*, one that kills both tumor and normal cells, which still is a major problem in conventional drug screening; and (d) it is able to detect differential pathways activated in normal versus tumor cells.

In the method described here, desirable chemopreventive/therapeutic agents induce apoptosis in the tumor cells (detected by diminished green fluorescence) and induce p57 expression (detected by red fluorescence) in normal cells concomitantly. The effects of an agent can be recorded by simple standard immunofluorescence microscopy techniques. To our knowledge, this model represents the first co-culture drug screening approach that monitors intracellular pathways for tumor cell destruction and normal cell survival simultaneously. This method has the potential to be modified for high-throughput screening. Therefore, plant-derived compounds, numbered in the tens of thousands,<sup>18</sup> could be efficiently screened for their anticancer properties. Further, the principles of the system are adaptable to other pathways and cell lines.



**FIG. 1.** Survival and apoptotic pathways activated by GTPPs/EGCG. GTPPs or EGCG activates separate pathways depending upon the cell type. In normal human epithelial cells such as NHEK, EGCG induces p57 expression, followed by induction of keratins, filaggrin and caspase 14 (a terminal differentiation factor), and inhibition of p21 expression (cyclin-dependent kinase involved in growth arrest, apoptosis, and differentiation), resulting in differentiation-associated cell survival (left). In many tumor cells, including OSC-GFP, a death signal is sent to the mitochondria, causing cytochrome *c* release and p21 expression, followed by the assembly of the apoptosome and activation of the caspase cascade, resulting in apoptosis. In this case, the apoptosis is associated with the loss of green fluorescence (right). If p57 is overexpressed, apoptosis is inhibited in the tumor cells.

## Materials and Methods

### Chemicals and antibodies

EGCG was purchased from Sigma (St. Louis, MO, U.S.A.). A mixture of four major GTPPs was purchased from LKT Lab., Inc. (Minneapolis, MN, U.S.A.). GTPPs and EGCG were dissolved in KGM-2 (Cambrex Corp., East Rutherford, NJ, U.S.A.) and filter-sterilized immediately prior to use. The rabbit anti-human p57 antibody and goat anti-rabbit IgG-rhodamine were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

### Cell lines and cell culture

Pooled normal human primary epidermal keratinocytes (NHEK) were obtained from Cambrex and maintained in KGM-2 medium (Cambrex). The OSC-2 cell line was isolated from cervical metastatic lymph nodes of a patient with oral squamous cell carcinoma,<sup>19</sup> and was cultured in DMEM/Ham's F12 50/50 mix medium (Cellgro, Kansas City, MO, U.S.A.) supplemented with 10% (vol/vol) fetal bovine serum, 100 IU/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin, and 5  $\mu\text{g/ml}$  hydrocortisone. OSC-2 has a missense mutation (exon 8, codon 280: AGA  $\rightarrow$  ACA) causing p53 overexpression.<sup>20</sup> The human lung diploid fibroblasts WI-38 were purchased from American Type Culture Collection and maintained in F12 medium supplemented with 5% Nu Serum, 125 units/ml penicillin, 125  $\mu\text{g/ml}$  streptomycin, and 10  $\mu\text{g/ml}$  glutamine.

### Generation of OSC-GFP cell lines

The GFP cDNA (Clontech, Palo Alto, CA, U.S.A.) was subcloned into the *Hind*III site of the retroviral vector pLNCX2 (Clontech). Virus was generated in RetroPack PT67 cells (Clontech) by transfection and antibiotic G418 selection. The transfected PT67 cells were cultured in standard DMEM. The viral titer was determined according to the manufacturer's suggestion. OSC-2 cells were transfected by incubation for 24 h with the virus-containing DMEM removed from PT67 culture. The GFP-expressing clones were selected by 60  $\mu\text{g/ml}$  G418.

### Co-culture and GTPP treatment

Various patterns of co-culture of the tumor/normal cells were achieved by different designs. Examples are listed below:

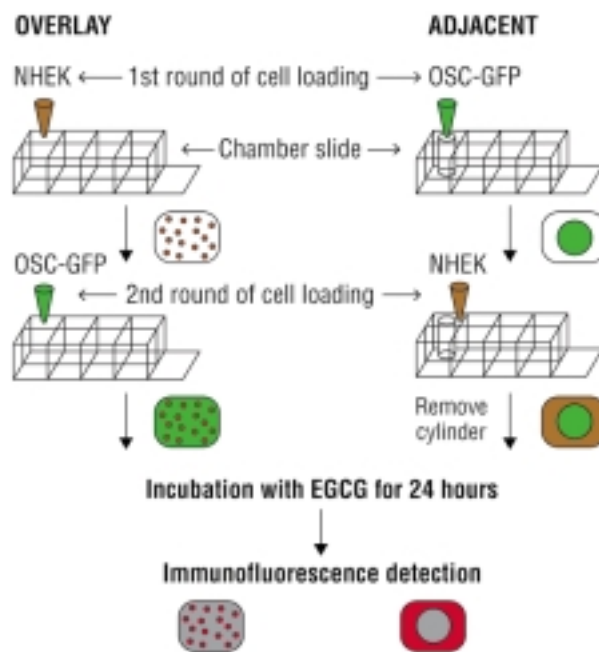
1. Adjacent co-culture design. OSC-GFP cells ( $5 \times 10^4$ ) were seeded in the center of a culturing device (eight-well chamber slide, Nalgel Nunc International, Naperville, IL, U.S.A.) through a cloning cylinder (Fisherscientific/Scienceware, tapered design,  $4.7 \times 8$  mm) in DMEM/F12 and allowed to attach for 24 h. NHEK ( $10^5$ ) in KGM-2 were then seeded in the area next to the cylinder. After 24 h, the cylinder was removed and the medium was replaced by a 50/50 mix of KGM-2 and DMEM/F12, and the cells were allowed to grow for another 24 h prior to treatment (Fig. 2, right).
2. Overlay design, which could be adapted for high throughput screening in 96-well plate. NHEK ( $2 \times 10^4$ ) were seeded in the wells of an eight-well chamber slide and allowed to grow in KGM-2 for 48 h. OSC-GFP cells ( $2 \times 10^5$ ) were then seeded in the wells in DMEM/F12 for 24 h. Medium was changed

to a 50/50 mix of KGM-2 and DMEM/F12 for 24 h prior to treatment (Fig. 2, left).

3. For monitoring co-culture of fibroblasts adjacent to OSC-2 cells, OSC-GFP cells ( $5 \times 10^4$ ) were seeded in the center of wells of an eight-well chamber slide through a cloning cylinder and incubated for 24 h. Human lung diploid fibroblasts WI-38 ( $10^5$ ) were then seeded in the area next to the cylinder in F12 medium and allowed to grow for 24 h. The cylinder was then removed, and the medium was changed to a 25/75 mix of DMEM/F12 for 24 h prior to GTPP treatment.
4. Tumor cell migration was monitored by co-culturing OSC-GFP and NHEK in wells of a 24-well plate as indicated in "1" above, except the NHEK cell number was doubled. After treatment, tumor cell migration into NHEK territory was recorded by fluorescent microscopy at selected time points (note: NHEK may be replaced by other normal cells, such as WI-38).

### Immunofluorescence and photography

At the end of a 24-h treatment with EGCG, the eight-well chamber slide was washed with PBS and fixed in a 4% paraformaldehyde/PBS solution for 30 min at room temperature, followed by washing with PBS three times.



**FIG. 2.** Procedures involved in different designs for co-cultures. The overlay design requires two rounds of loading of cells. Cells loaded in the second round cover the cells loaded in the first round (left). The adjacent design also requires two rounds of cell loading, but the two cell types are separated by a cylinder (right). After the co-cultures are treated with EGCG, the slide is subjected to fixation and immunofluorescence, followed by rhodamine and GFP detection and quantification.

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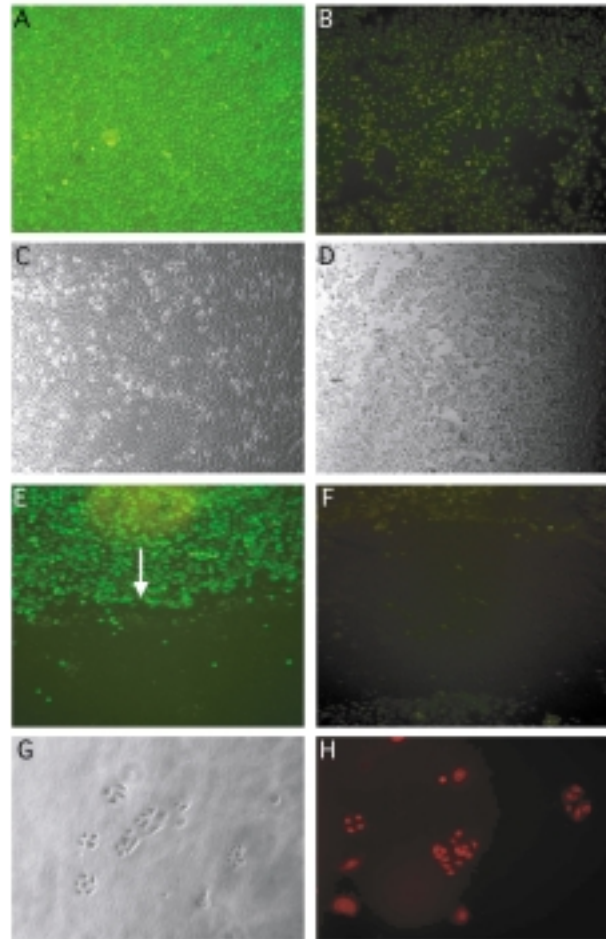
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The slide was then treated with permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) on ice for 2 min, followed by PBS washing for three times. The slide was incubated in blocking buffer (5% goat serum and 5% BSA in PBS) at 37°C for 60 min. The primary antibody, rabbit anti-human p57 polyclonal antibody (H91, Santa Cruz) in PBS/5% BSA, was applied to the samples for 1 h at 37°C at the dilution (1:50) recommended by the manufacturer. Negative control sections consisted of cells incubated with 1% diluted normal goat serum instead of primary antibody. After washing three times with PBS, the slide was incubated with the secondary anti-rabbit IgG conjugated with rhodamine (Santa Cruz) for 1 h at 37°C. Finally, the slide was washed three times with PBS containing 0.1% Tween-20, mounting solution (Prolong Antifade, Molecular Probes, Eugene, OR, U.S.A.) was applied to each well, and the slide was covered by a cover slip. The samples were visualized under a Nikon Phase Contrast-2 microscope. Fluorescent photomicrographs were taken with a SPOT color digital camera system (Diagnostic Instruments) using the Zeiss Axiovert 10 with an original magnification of 200×. Fluorescence was generated by a Zeiss AttoArc 2 source. Light photographs were taken with a SPOT RT digital camera system linked to a Nikon Phase Contrast-2 microscope at an original magnification of 200×.

The total fluorescence intensities of images were quantified using the BIOQUANT NOVA PRIME 6.0 software (Bioquant Co., Nashville, TN, U.S.A.). The ratio of rhodamine/FITC reflects the status of the p57-associated survival pathway in NHEK and the apoptosis pathway in OSC-GFP in the normal/tumor co-culture.

## Results

A retroviral promoter-driven, GFP-expressing OSC-2 cell line (OSC-GFP) was previously generated.<sup>7</sup> This cell line maintains the parental line's high sensitivity to GTPP-induced apoptosis at concentrations encountered by the oral mucosa (up to 0.3 mg/ml), and shows diminished green fluorescence associated with apoptosis (Fig. 3A–D). Results from cell growth and caspase 3 activity assays showed that OSC-GFP cells responded to GTPPs or EGCG similar to the parental OSC-2 cells (data not shown). When OSC-GFP cells were co-cultured with NHEK cells, as shown by the reduction in green fluorescence, GTPPs induced apoptosis at a level comparable to that seen in OSC-GFP cells alone (Fig. 3E and F), indicating that co-culture or the mix of culture media did not alter the signal for apoptosis in OSC-GFP cells when exposed to GTPPs. Induction of p57 by EGCG in NHEK grown alone was confirmed by immunofluorescence (Fig. 3G and H). Co-culture with OSC-GFP cells did not affect the induction (see Fig. 4E and F). The red fluo-



**FIG. 3.** Results of cell death and survival experiments. (A) GFP-OSC-2 cells grown alone showed bright green fluorescence when cultured without GTPPs. (B) The green fluorescence was lost after 48-h exposure to 0.2 mg/ml GTPPs, followed by growth in normal medium for an additional 48 h. (C) Optical microscopy view of the same fields as in A. (D) Extensive cell death is apparent only in GTPP-treated cells, resulting in morphological changes visible by light microscopy. (E) FITC fluorescent microscopy of GFP-OSC-2 cells bordering NHEK grown for 96 h. The arrow points to the border area. (F) FITC fluorescent microscopy showing GFP-OSC-2 cells bordering NHEK treated with 0.2 mg/ml GTPPs for 48 h, and placed in normal medium for additional 48 h. (G) Light microscopy showing NHEK grown alone after 24-h treatment with 100  $\mu$ M EGCG. No cell death was observed. (H) Rhodamine fluorescent microscopy viewing NHEK alone treated with 100  $\mu$ M EGCG for 24 h, followed by immunofluorescence staining with p57 primary antibody and secondary antibody conjugated with rhodamine.

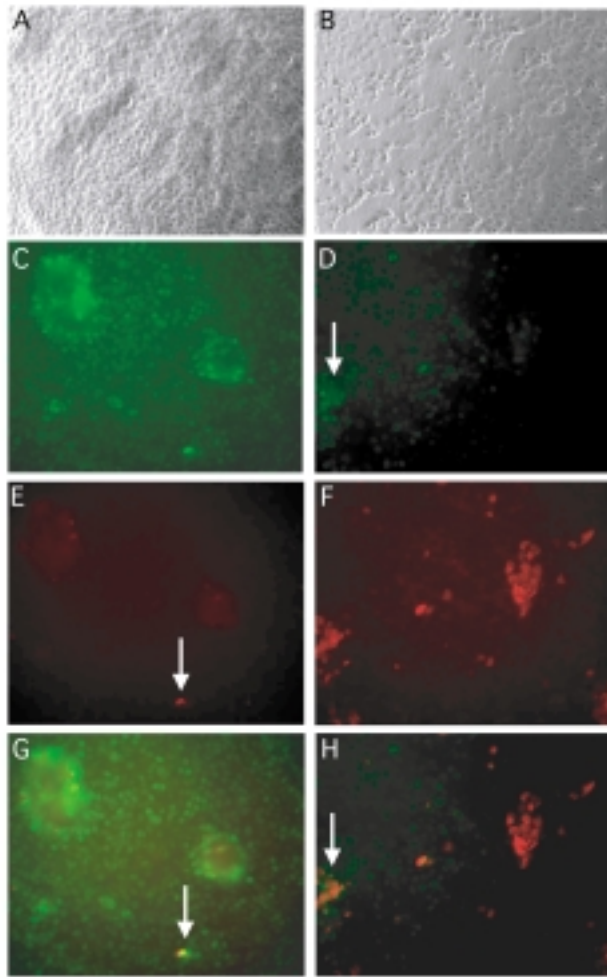
rescence was therefore used as a cell survival indicator in the co-culture system.

When OSC-GFP cells and NHEK were plated in an overlay pattern, EGCG exposure resulted in extensive apoptosis in OSC-GFP cells, leaving large unoccupied spaces (Fig. 4A and B). Untreated co-culture cells exhibited strong green fluorescence compared with EGCG-

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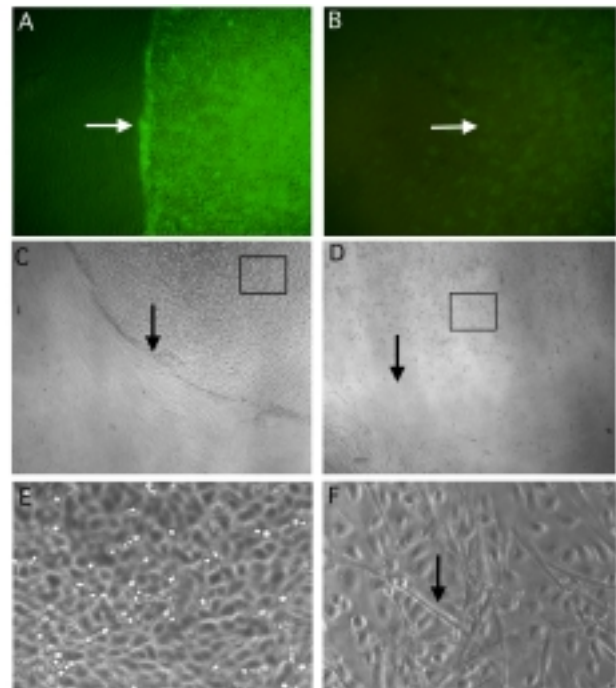
**FIG. 4.** Overlay design of the co-culture of NHEK and OSC-GFP. Left panels represent untreated cells. (A) Light microscopy of OSC-GFP seeded over NHEK colonies incubated for 24 h in medium without EGCG. (C) Same area as in A photographed by FITC fluorescent microscopy. (E) Rhodamine fluorescent microscopy after p57 immunofluorescence staining of NHEK. One keratinocyte expressed p57 as indicated by arrow. (G) Merged image of C and E. Right panels show the co-cultured cells treated with 200  $\mu$ M EGCG for 24 h. (B) Light microscopy of the morphological changes of the co-culture. (D) FITC fluorescence in the same area as in B. Few viable OSC-GFP cells were present, as indicated by arrow. (F) p57 immunofluorescence staining of NHEK demonstrated p57 induction in NHEK colonies. (H) Image merged from D and F.

treated co-culture cells (Fig. 4C and D), whereas p57 induction was only detected in EGCG-treated co-culture cells, as indicated by rhodamine (red fluorescence) in NHEK (Fig. 4E and F). Green/red merged images demonstrated OSC-GFP cells expressing strong GFP, whereas NHEK only express basal p57 without EGCG exposure (Fig. 4G). In contrast, EGCG-treated co-culture showed an opposite pattern compared with untreated: strong red fluorescence and diminished green fluorescence, representing simultaneously tumor cell apoptosis

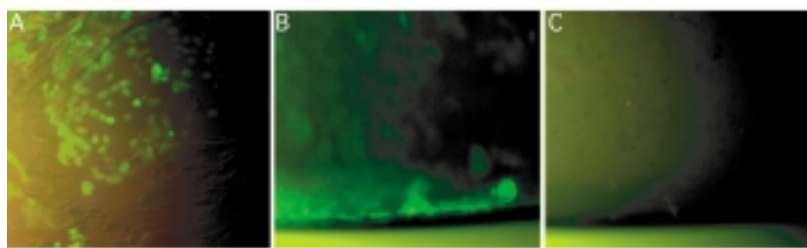
and NHEK survival (Fig. 4H). Quantitative measurement using the BIOQUANT NOVA PRIME 6.0 software showed that the ratio of fluorescence intensities of rhodamine (red)/FITC (green) in the control cells (*e.g.*, Fig. 4G) was 0.01, whereas in the EGCG-treated cells (*e.g.*, Fig. 4H) it was 1.23. That is, there was a more than 100-fold change in the relative ratios following EGCG treatment.

When OSC-GFP cells were plated adjacent to WI-38 cells, untreated co-culture cells exhibited a defined border between the two cell types observed by either fluorescent microscopy (Fig. 5A) or light microscopy (Fig. 5C). A clear border was not formed in the co-culture treated with GTPPs due to tumor cell apoptosis (Fig. 5B and D). OSC-GFP cells without GTPP treatment were able to expand into the WI-38 occupied area (Fig. 5C) and did not allow WI-38 cell infiltration (Fig. 5E). GTPPs

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**FIG. 5.** Co-culture of WI-38 human fibroblasts and OSC-GFP cells. Left panels show untreated co-culture for 96 h. (A) Fluorescent microscopy of the border area (indicated by arrow) between two cell types. (C) Light microscopy of the co-culture showing border between the two cell types indicated by arrow. (E) Enlarged image from the framed area in C, no fibroblast infiltration. Right panels present the co-culture post treatment. (B) FITC fluorescent microscopy showing diminished green fluorescence, indicating death of OSC-GFP cells. Arrow points to the gap between tumor and normal cells. (D) Light microscopy after 48-h GTPP treatment (0.2 mg/ml). No apparent border between WI-38 cells and OSC-GFP cells is seen. The arrow points to the gap area. (F) Enlarged image from the framed area in D. An infiltration of WI-38 cells occupied the areas where OSC-GFP cells were destroyed.



**FIG. 6.** Real-time monitoring of tumor cell migration. (A) GFP-OSC-2 cells without GTPP treatment migrated into NHEK (dark area). (B) Distance migration of GFP-OSC-2 cells without GTPPs. These cells have reached the edge of the well. (C) When GTPPs were present for 48 h, no tumor cells were found in the edge area. The green background in the lower portion is from the fluorescent light source (FITC).

caused both OSC-GFP cell apoptosis and WI-38 cell infiltration, seen as elongated fibroblasts (arrow, Fig. 5F).

When OSC-GFP cells were plated adjacent to NHEK, the tumor cells migrated onto the layer of NHEK (Fig. 6A). The tumor cells reached the edge of the well in 48 h (Fig. 6B). In contrast, OSC-GFP cells in GTPP-treated co-culture failed to migrate (Fig. 6C).

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## Discussion

Many leafy plants, either fruits or vegetables, have high levels of phenolic compounds.<sup>21,22</sup> These compounds are part of the plants' defense system, acting as pesticides against a variety of organisms. However, primates closely related to humans rely predominantly on fresh leafy plants for their energy needs. It is likely that primates, including humans, may have evolved a tolerance to exposure to these phenolic compounds in the epidermis, oral epithelium, and digestive tract. High concentrations of tea polyphenols (50–600  $\mu\text{M}$ ) not only failed to induce cell damage in these tissues, but also provide protection against reactive oxygen species.<sup>1,23–26</sup> NHEK have been widely reported to tolerate high concentrations of tea polyphenols.<sup>27,28</sup> The survival mechanism of NHEK involves cell differentiation associated with p57 induction, which is time- and dose-dependent.<sup>6,7</sup> In contrast, normal cells derived from internal organs, such as bronchial, mammary, and kidney, can be damaged by polyphenols and undergo apoptosis at concentrations higher than the maximal plasma concentration.<sup>12,29–31</sup> Caspase 3 positive tumor cells, such as the oral carcinoma lines OSC-2 and SCC25 and the breast carcinoma T47D cell line, as well as caspase 3-transfected MCF7 cells, also undergo a caspase 3-dependent apoptosis upon exposure to the polyphenols.<sup>6,7,12</sup> Transfection and expression of p57 cDNA in OSC-2 cells resulted in resistance to GTPP-induced apoptosis.<sup>7</sup> Therefore, in the current study, p57 expression was chosen as a marker for activation of a cell survival pathway, whereas well characterized OSC-2 cells were chosen to reflect polyphenol-induced apoptosis (Fig. 3). Other normal/tumor cell systems may also be adapted for drug-screening purposes according to specific needs, but we recommend using normal cells that can be induced by

phenolic compounds to express a large amount of p57 or caspase 14, a terminal differentiation marker, which is overexpressed after GTPP treatment (data not shown, manuscript in preparation). Tumor cells that either express high levels of p57 or lack functional caspase 3 should be avoided because they might be resistant to the effects of phenolic compounds.<sup>12</sup> The designs of devices to be used for the co-culture system are very flexible, depending on the purpose of the testing. An eight-well chamber slide was used in the current study for double fluorescence detection as shown in Fig. 4; images in the left panel were taken from a single area of untreated co-culture, which shows light microscopy (Fig. 4A), FITC fluorescence (Fig. 4C), rhodamine fluorescence (Fig. 4E), and merged fluorescent images (Fig. 4G). Compared with images of EGCG-treated co-culture cells (Fig. 4, right panels), the differential effects of EGCG were apparent, especially in the merged images (Fig. 4G and H). Combined with paired light microscopy images, interpretation of results can be simplified as follows: (a) if an agent causes diminished green fluorescence and induces red fluorescence, this agent is able to destroy tumor cells while protecting normal cells; (b) if an agent causes diminished green fluorescence but does not induce red fluorescence, this agent is able to destroy tumor cells but not protect normal cells; (c) if an agent does not cause diminished green fluorescence nor induce red fluorescence, this agent is not able to kill tumor cells or protect normal cells; (d) if an agent induces red fluorescence but does not diminish green fluorescence, this agent is able to protect normal cells but does not destroy tumor cells. As shown above, there is a large (>100-fold) increase in the relative red:green ratio in the co-cultured wells following EGCG treatment. This large difference represents the simultaneous induction of survival-associated expression of p57 (tagged by rhodamine) and apoptosis-associated reduction of GFP (measured by FITC filter) after the co-culture was treated with 100  $\mu\text{M}$  EGCG (a level well within the range that oral epithelial cells could be exposed to under normal dietary conditions).

One strategy to adapt this approach to high throughput screening will be to use a dual-fluorescence microplate reader to measure quantitatively the differential effect of candidate agents in a 96-well plate format, for example, by measurement of the ratio of total rho-

damine/FITC fluorescence per well, as described above. The overlay method described above will be the simplest to adapt to this format.

In addition to identifying the differential effects of potential anticancer agents, which promote normal epithelial cells to enter a survival/differentiation pathway and tumor cells to enter an apoptotic pathway, as shown in Fig. 5, this system is also able to test the impact of a given agent on tumor/normal cell interaction. The untreated co-culture of OSC-GFP and WI-38 cells demonstrated tumor cell expansion toward the fibroblasts (Fig. 5A and C). The border area exhibited physical pressure from tumor cells (arrows), and there were no fibroblasts found among tumor cells (Fig. 5E). These characteristics were not observed in GTPP-treated co-culture, where the border was not formed (arrows, Fig. 5B and D). During the treatment time when the tumor cells underwent apoptosis, the fibroblasts migrated and infiltrated into the area previously occupied by the tumor cells (Fig. 5F), suggesting that cell movement from the opposite direction occurred. What attracted the fibroblast migration is unknown. We have reported that EGCG inhibited OSC-2 cell invasion and migration in transwells without other cell types.<sup>12</sup> The current study confirmed this previous observation (Fig. 6A). Therefore, the co-culture system is adequate to test a given agent for its antimigration potential by real-time monitoring and recording of tumor cell movement compared with untreated co-culture (Fig. 6B and C). In conclusion, this mechanism-based *in vitro* co-culture system could be used to screen plant-derived phenolic compounds, and other agents, for their differential effects toward apoptosis and survival with simple detection methods and flexible designs. High throughput screening can be achieved with certain modifications. In addition to drug screening, cell interaction and tumor cell migration can be monitored by this system.

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**HSU**

**AU1**

**Is change to Clontech as meant 3x?**

**AU2**

**“X-100” as meant?**

**AU3**

**“GTPPs” as meant?**