Molecules and Cells

Minireview

Cancer Prevention with Green Tea and Its Principal Constituent, EGCG: from Early Investigations to Current Focus on Human Cancer Stem Cells

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Cancer preventive activities of green tea and its main constituent, (-)-epigallocatechin gallate (EGCG) have been extensively studied by scientists all over the world. Since 1983, we have studied the cancer chemopreventive effects of EGCG as well as green tea extract and underlying molecular mechanisms. The first part of this review summarizes groundbreaking topics with EGCG and green tea extract: 1) Delayed cancer onset as revealed by a 10-year prospective cohort study, 2) Prevention of colorectal adenoma recurrence by a double-blind randomized clinical phase II trial, 3) Inhibition of metastasis of B16 melanoma cells to the lungs of mice, 4) Increase in the average value of Young's moduli, i.e., cell stiffness, for human lung cancer cell lines and inhibition of cell motility and 5) Synergistic enhancement of anticancer activity against human cancer cell lines with the combination of EGCG and anticancer compounds. In the second part, we became interested in cancer stem cells (CSCs). 1) Cancer stem cells in mouse skin carcinogenesis by way of introduction, after which we discuss two subjects from our review on human CSCs reported by other investigators gathered from a search of PubMed, 2) Expression of stemness markers of human CSCs compared with their parental cells, and 3) EGCG decreases or increases the expression of mRNA and protein in human CSCs. On this point, EGCG inhibited self-renewal and expression of pluripotency-maintaining transcription factors in human CSCs. Human CSCs are thus a target for cancer prevention and treatment with EGCG and green tea catechins.

Keywords: AFM, Nanog, Oct4, Sox2, stemness

INTRODUCTION

The term "cancer chemoprevention" was introduced by Michael B. Sporn, at the US National Institutes of Health (NIH) in Bethesda, Maryland (Sporn et al., 1976), and Japanese cancer researchers became interested in screening for possible cancer preventive agents in the 1980s. We assumed that inhibitors of tumor promotion would be cancer preventive compounds, since they had been known to suppress experimentally induced tumor development in rodents (Boutwell, 1977). Since green tea is a daily beverage in Japan, we paid special attention to green tea catechins, especially its main constituent (-)-epigallocatechin gallate (EGCG), for our main experiments (Fujiki and Okuda 1992). In 1987, we reported for the first time that topical applications of EGCG significantly prevented tumor promotion in mouse skin induced by teleocidin, one of the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoters (Yoshizawa et al., 1987). Soon after publication, the British journal "New Scientist" introduced our research on cancer prevention with EGCG under the title of "Green tea cuts cancerous growths" (Editor, 1987). In quick response to this article, staff members of the Australian TV scientific series "Beyond 2000" visited us in Tokyo: They made a film about our experiments in cancer

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prevention with green tea catechins, and the film was released in Australia, U.S.A., England and Europe in 1988. It was followed by "Beyond Tomorrow" on American TV in 1988. Many overseas scientists then became greatly interested in cancer prevention with green tea, some of them even more interested than Japanese scientists.

Since then, numerous scientists have shown that EGCG and green tea extract in drinking water prevent carcinogenesis in various organs of rodents (Conney et al., 1992; Fujita et al., 1989; Fujiki and Suganuma, 2002; Gupta et al., 2001; Surh 2003; Yamane et al., 1995; Yang et al., 2009; Yoshizawa et al., 1992). The results are supported by our experiments showing that intubation of ^{3}H-EGCG into mouse stomach distributed radioactivity in a wide range of target organs, and subsequent treatment of cells with ^{3}H-EGCG showed radioactivity inside the cells (Okabe et al., 1997; Suganuma et al., 1998). Based on results showing that the inhibitory effects of EGCG and green tea extract on the growth of human lung cancer cell lines PC-9 and PC-14 were approximately 250-fold less effective than adriamycin (Komori et al., 1993), we conceived the development of EGCG and green tea extract as cancer preventives, rather than cancer therapeutic drugs.

We have studied cancer prevention with green tea for over 30 years, and our collaborations have produced numerous significant results, both from basic studies and with cancer patients and the general human population (Fujiki et al., 2002; 2012). This review consists of two parts, and the first part summarizes epochal results by way of introduction: 1) A prospective cohort study revealed that drinking 10 Japanese-size cups (120 ml/cup) of green tea per day delayed cancer onset 7.3 years for female patients (Imai et al.,1997; Nakachi et al., 2000); 2) A randomized phase II clinical prevention trial showed that drinking 10 Japanese-size cups of green tea, supplemented with green tea tablets, significantly reduced tumor recurrence in patients with colorectal adenomas (Shimizu et al., 2008); 3) Peroral administration of EGCG in drinking water prevented both hematogenous and lymphogenous (spontaneous) lung metastases of B16 melanoma cells in male C57BL/6 mice (Taniguchi et al., 1992); 4) Treatment of B16-F10 mouse melanoma cells with EGCG increased the average value of Young's moduli as assessed by the atomic force microscope (AFM), i.e., cell stiffness, and inhibited cell motility (Watanabe et al., 2012); and 5) The combination of EGCG and anticancer compounds induced apoptosis and increased efficacy of anticancer activity in rodents, and also showed synergistic enhancement of anticancer activity against human cancer cell lines (Suganuma et al., 1999; 2006; 2011). Green tea is a cancer preventive for primary cancer prevention, and green tea catechins act as synergist with anticancer drugs in tertiary cancer prevention (Fig. 1) (Fujiki, 2017; Fujiki et al., 2012; 2015a; 2015b).

The second part of this review shows the molecular mechanisms of anticancer activity of EGCG against human cancer stem cells (CSCs). We first found that repeated applications of 5 mg EGCG before each treatment with 1 μg okadaic acid - a potent tumor promoter and inhibitor of protein phosphatases 1 and 2A - completely prevented tumor promotion in mouse skin, in two-stage carcinogenesis experiments initiated with 7,12-dimethylbenz(a)anthracene (DMBA), and that treatment with DMBA plus okadaic acid produced tumors in 73.3% of mice at week 20 (Fujiki and Suganuma, 1993; Suganuma et al., 1988). We believe that EGCG treatment inhibited the interaction of tumor promoters, such as okadaic acid, TPA and teleocidin with their receptors, and theorized that EGCG can interrupt the interaction of ligand with its receptor on cell membrane. This is now called the "Sealing effects of EGCG" (Fig. 2) (Yoshizawa et al., 1992): We thus decided to study cancer stem cells in mouse skin as a target of cancer prevention. In 2012, Srivastava's group reported that EGCG inhibited viability of human pancreatic CSCs in primary and secondary spheroids, as well as expression of pluripotency-maintaining factors in the CSCs (Tang et al., 2012). Recently we published a review article on the anticancer activity of EGCG against various human CSCs enriched from cancer cell lines (Fujiki et al., 2017). We then discuss: 1) Cancer stem cells in mouse skin carcinogenesis, 2) Expression of stemness markers of human CSCs enriched from colorectal and nasopharyngeal cancer cell lines, compared with their parental cells, and 3) Decrease or increase in the expression of mRNA and protein in human CSCs of breast, lung and colorectal cancers after treatment with EGCG. The inhibitory effects of EGCG on self-renewal and expression of transcription factors in human CSCs derived from various cancer tissues are emphasized. It has now become clear that human CSCs are effective targets for prevention and treatment using EGCG and green tea extract.

DELAYED CANCER ONSET REVEALED BY 10-YEAR PROSPECTIVE COHORT STUDY

Nakachi and Imai found a total of 419 cancer patients, 175 females and 244 males, during 10 years, from a survey living habits of 8,552 individuals aged over 40 living in Saitama Prefecture, including their daily consumption of green tea (Imai et al., 1997; Nakachi et al., 2000). They reported that cancer onset in female patients who had consumed over 10 Japanese-size cups (120 ml/cup) of green tea per day was 7.3 years later than that of patients who had consumed less than three cups per day (Table 1). The difference between females and males may be partly due to higher tobacco consumption by males. Consuming over 10 cups of green tea per day (corresponding to 2.5 g green tea extract) also significantly prevented lung cancer, with a relative risk of 0.33,

Table 1. Daily green tea consumption and average age at cancer onset*

Gender	Daily consumption of green tea (cups)		
	≤3	4 - 9	≥10
	Average age at cancer onset (% of patients)		
Female (175)	67.0 ± 1.7	66.4 ± 1.3	74.3 ± 2.2
	(28.0%)	(58.3%)	(13.7%)**
Male (244)	65.0 ± 1.5	67.2 ± 1.0	68.2 ± 1.1
	(24.2%)	(46.7%)	(29.1%)

*All different types of cancer are included, ** $P < 0.01$

Table 2. Recurrence rate of breast cancer in relation to daily consumption of green tea

Parameter	Daily green tea consumption ≤4 cups	≥5 cups
Stages I and II (390 patients)		
Recurrence rate (%)	24.3	16.7*
Disease-free period (years)	2.8	3.6
Stage III (82 patients)		
Recurrence rate (%)	48.8	58.5
Disease-free period (years)	1.9	1.9

*$P < 0.05$ in terms of the Cox proportional hazards model.

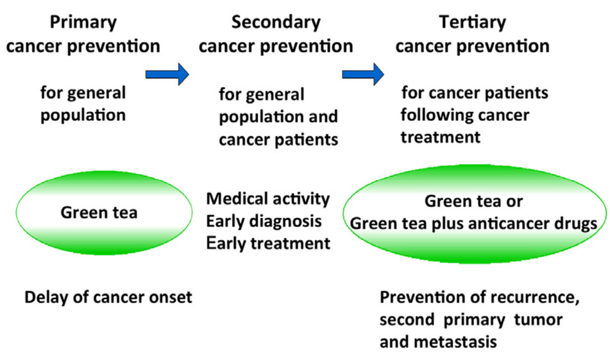

Fig. 1. Primary, secondary, and tertiary cancer prevention in humans.

followed by cancers of the colorectum, liver and stomach, in that order (Imai et al., 1997; Nakachi et al., 2000). Results of two research groups: Green tea catechins at 600 mg per day were effective for treating premalignant lesions before prostate cancer development, research conducted at University of Parma, Italy (Bettuzzi et al., 2006): and green tea extract showed preventive effects on oral premalignant leukoplakia in patients, at the University of Texas M.D. Anderson Cancer Center (Tsao et al., 2009).

Nakachi and Imai also studied recurrence of breast cancer among the 472 cancer patients: Stages I and II cancer patients consuming over five cups of green tea per day (average 8 cups) showed a lower recurrence rate, 16.7%, and a longer disease-free period, 3.6 years, than those consuming less than four cups per day (average 2 cups), 24.3% and 2.8 years (Table 2) (Nakachi et al., 1998). However, in Stage III breast cancer patients, green tea did not show any decreased recurrence because Stage III breast cancer includes more accumulated genetic changes in the cells than are found in Stages I and II. The results were later confirmed at Aichi Cancer Center (Inoue et al., 2001), and at Harvard T.H. Chan School of Public Health in the United States (Ogunleye et al., 2010).

Since humans are always at risk of tumor promotion induced by inflammation, we need to establish a cancer prevention strategy that can reduce TNF-α, IL-1 and other proinflammatory cytokines, and inactivate NF-κB (Fujiki et al., 2013). The key point is: Drinking green tea contributes to primary cancer prevention (Fig. 1).

PREVENTION OF COLORECTAL ADENOMA RECURRENCE WITH 10 JAPANESE-SIZE CUPS OF GREEN TEA PER DAY SUPPLEMENTED WITH GREEN TEA TABLETS

The Saitama Prefectural Tea Research Institute began to produce tablets of green tea extract (G.T.E), which is the dried form of green tea beverage. One tablet is equivalent to approximately 2 Japanese-size cups of green tea. One hundred two healthy citizens of Saitama Prefecture joined the preclinical safety trial of G.T.E, with informed consent. The blood examination did not show any serious effects, and 93% of the participants were able to continue drinking green tea beverage and also taking G.T.E (Fujiki et al., 2001). Because some of the subjects had very mild temporary disorders, the Tea Research Institute subsequently reduced the caffeine content of the tablets from 5% to less than 3% without using an organic solution.

Moriwaki's group at Gifu University conducted a double-blind randomized clinical phase II prevention trial of colorectal adenoma recurrence with subjects drinking 10 cups of green tea supplemented with tablets of G.T.E, with informed consent. Patients without colon adenomas were then double-blind randomized into two groups: Control group maintained daily consumption of green tea beverage only, without a placebo, and G.T.E group took the daily beverage plus 3 tablets (equivalent to 6 cups) per day, corresponding to over 10 cups, about 2.5 g green tea extract, for 12 months. The incidence of recurrent adenomas was determined by end-point colonoscopy 12 months later: Control group showed 31.0% recurrence rate, while the G.T.E group rate was 15.0%, and the average size of relapsed adenomas was 3.0 ± 1.0 mm in the G.T.E. group and 4.0 ± 1.3 mm in control group ($P < 0.001$) (Table 3). Thus, drinking 10 Japanese-size cups of green tea, supplemented with G.T.E, significantly, 51.6%, reduced recurrence of colorectal adenomas (Shimizu et al., 2008).

Table 3. Phase II prevention trial of colorectal adenoma recurrence of patients drinking a combination of daily green tea beverage and tablets of G.T.E

Study in Japan		
Groups (cases)	Recurrence rate %	Size of relapsed adenomas (mm)
Control (20/65)	31.0	4.0 ± 1.3
G.T.E (9/60)	15.0*	3.0 ± 1.0**
Study in Korea		
Groups (cases)	Recurrence rate %	Size of the largest polyps (mm)
Control (43/71)	60.6	4.8 ± 2.4
G.T.E (20/72)	27.8**	4.8 ± 2.3

*$P < 0.05$, **$P < 0.001$

Similar results were confirmed at different institutions: drinking green tea extract prevented 44.2% of colorectal adenoma recurrence in Korean patients at Seoul National University (Table 3) (Shin et al., 2017), and the flavonoid mixture (daily standard dose, 20 mg apigenin and 20 mg EGCG) reduced the recurrence rate of colon neoplasia in patients with resected colon cancer at the Hospital of Gross-Gerau, Germany (Hoensch et al., 2008). And a plan for the first large-scale placebo-controlled prevention trial for metachronous adenoma recurrence in the colorectum of patients, using green tea extract for three years, is conducted at University Ulm, Germany (Stingl et al., 2011). All the results show that drinking green tea is effective for tertiary cancer prevention (Fig. 1).

INHIBITION OF METASTASES OF B16 MELANOMA CELLS TO THE LUNGS OF MICE BY DRINKING EGCG

Taniguchi's group reported for the first time that oral administration of EGCG inhibited lung metastases of two different B16 melanoma variants in two experimental models (Taniguchi et al., 1992). Hematogenous metastasis was induced with intravenous injection of highly metastatic B16-F10 cells in male C57BL/6 mice given a solution of 0.05% and 0.1% EGCG. Lymphogenous (spontaneous) metastasis was induced by inoculation of highly metastatic and invasive B16-BL6 cells into the right foot pads of male C57BL/6 mice, given the same solutions of EGCG. EGCG reduced the average number of lung nodules (Table 4) (Taniguchi et al., 1992). To understand the inhibitory effects of EGCG on metastasis, Suganuma's group further studied the biophysical effects of EGCG on cell stiffness and motility (Suganuma et al., 2016).

GREEN TEA AND EGCG INCREASED THE AVERAGE VALUE OF YOUNG'S MODULUS OF CANCER CELLS (CELL STIFFNESS) AND INHIBITED CELL MOTILITY

Cell stiffness can be determined using atomic force microscope (AFM), which quantitatively provides the average value of Young's modulus of cancer cells. In 2007, Gimzewski's group at UCLA reported that metastatic cells in pleural fluids obtained from lung, breast, and pancreatic cancer patients have significantly lower average values of Young's modulus with less stiffness (equivalent to smaller elasticity) - determined using AFM - than normal mesothelial cells in the body fluids (Cross et al., 2007). They also found that green tea extract dramatically increased cell stiffness of metastatic cancer cells, from 0.43 kPa to 2.53 kPa, about 6.2-fold, based on the average value of Young's moduli in nine cancer cell lines (Cross et al., 2011). The results indicated that AFM can measure the changes in cell stiffness induced by EGCG.

To study the relationship between increased average value of Young's moduli and reduction of cell motility by Transwell assay, we used three metastatic B16 mouse melanoma variants for experiments: B16-F10 cells are most motile, B16-BL6 cells, medium motile and B16-F1 cells, least motile. Young's modulus of the most motile B16-F10 cells showed significantly lower cell stiffness, i.e., more soft elasticity, than those of B16-BL6 cells and B16-F1 cells. Furthermore, treatment of B16-F10 cells with 100 μM EGCG increased the average value of Young's modulus to 0.68 ± 0.03 from 0.44 ± 0.01 kPa of non-treated B16-F10 cells (Table 5). The results indicated that EGCG increased the average value of Young's modulus for B16-F10 cells (0.68 kPa), which was comparable to that of the least motile B16-F1 cells (0.72 kPa) without EGCG, showing that treatment with EGCG increased stiffness. Moreover, treatment of B16-F10 cells with EGCG (50 - 200 μM) dose-dependently reduced the motility of the cells to 57.1, 30.3 and 12.6%, respectively (Table 5), without affecting viability of the cells (Watanabe et al., 2012). Thus, EGCG simultaneously increased cell stiffness and enhanced inhibition of cell motility (Fig. 2).

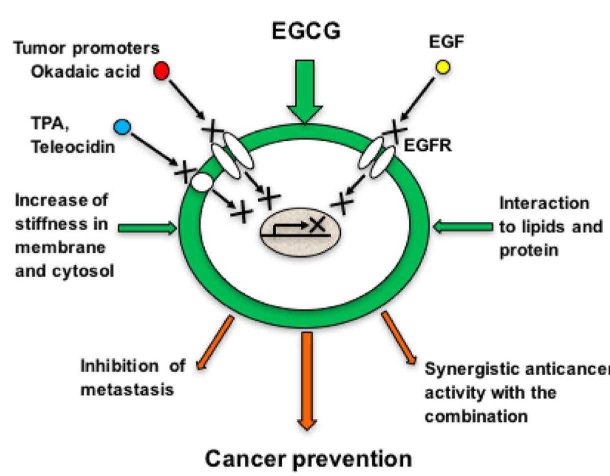

Fig. 2. Schematic illustration of mechanisms of EGCG in relation to "Sealing effects of EGCG".

Table 4. Inhibition of lung metastasis of B16 melanoma cells with peroral administration of EGCG

Groups	Hematogenous metastasis with B16-F10 cells		Lymphogenous metastasis with B16-BL6 cells	
	Average number of lung nodules	% of inhibition	Average number of lung nodules	% of inhibition
Control	>150		25	
0.05% EGCG	107*	>29%	7*	72%
0.1% EGCG	76*	>50%	10*	60%

*$P < 0.01$

Table 5. Average values of Young's moduli and inhibition of cell motility for B16-F10 mouse melanoma cells

	EGCG (μM)			
	0	50	100	200
Young's moduli (kPa)	0.44 ± 0.01	0.58 ± 0.03*	0.68 ± 0.03*	0.80 ± 0.02*
Cell motility (%)	100	57.1	30.3*	12.6**

*$P < 0.001$, **$P < 0.0001$

Table 6. Increase of average values of Young's moduli for human lung cancer cell lines by treatment with EGCG

	EGCG (μM)		
Cell lines	0	5	50
H1299 (kPa)	1.24 ± 0.05	2.30 ± 0.07*	2.25 ± 0.11*
Lu99 (kPa)	1.29 ± 0.11	1.63 ± 0.08*	2.28 ± 0.09*

*$P < 0.0001$

Suganuma's group found that treatment of human non-small cell lung cancer cell lines H1299 and Lu99 with EGCG (5 - 50 μM) for 4 h significantly increased the average value of Young's moduli from 1.24 ± 0.05 to 2.25 ± 0.11 kPa in H1299 cells, and from 1.29 ± 0.11 to 2.28 ± 0.09 kPa in Lu99 cells, showing a 2-fold increase of cell stiffness (Table 6) (Suganuma et al., 2016; Takahashi et al., 2014). The results showed that EGCG reduces membrane fluidity - increases rigidification of cell membrane - the cell stiffness of H1299 and Lu99, indicating that EGCG can reduce highly metastatic potential of both cell lines (Fig. 2).

SYNERGISTIC ENHANCEMENT OF ANTICANCER ACTIVITY AGAINST HUMAN CANCER CELL LINES WITH THE COMBINATION OF EGCG AND ANTICANCER COMPOUNDS

In 2011, Suganuma's group published a review article entitled "New cancer treatment strategy using combination of green tea catechins and anticancer drugs" in Cancer Sci. (Suganuma et al., 2011). The Publisher, Wiley-Blackwell at the Annual Meeting of Japanese Cancer Association, announced that our review article was No. 1 among most read articles and No. 2 among most cited articles in 2012. Since then, numerous scientists around the world have become greatly interested in the combination. We briefly showed that the combinations of EGCG or other green tea catechins and 46 anticancer drugs all synergistically induced *in vitro* anticancer effects in 58 human cancer cell lines (Fujiki et al., 2015a; 2015b).

It is important to note that the enhanced anticancer activity of the combination was demonstrated by reduction of tumor volume in xenograft mouse models in 13 *in vivo* experiments: human cancer cell lines from head and neck, lung, breast, prostate, liver, and stomach were implanted in experiments conducted by numerous investigators (Fujiki et al., 2015a; 2015b). It is striking that the combinations of EGCG and paclitaxel, and EGCG and docetaxel, completely eliminated tumors of human prostate cancer cell line PC-3ML *in vivo* (Table 7) (Stearns and Wang, 2011). In addition, average reduction of tumor volume (% of control) for the groups treated with vehicle (control), EGCG alone, anticancer drugs alone, and combinations were 100%, 73.5%, 66.3%, and 29.7%, respectively (Table 7). Thus, the combinations of EGCG and anticancer drugs significantly and synergistically reduced tumor volume by 70.3%, while treatment with EGCG or green tea extract alone was slightly less effective than that with anticancer drugs alone. When the amount of EGCG necessary for the complete elimination of tumors in mice is converted to that for humans, it would be 6 - 9 Japanese-size-cups of green tea, i.e., 1.37 - 2.05 g EGCG/day/person (Fujiki, 2017; Fujiki et al., 2015a; 2015b).

CANCER STEM CELLS IN MOUSE SKIN CARCINOGENESIS

Two-stage chemical carcinogenesis in mouse skin, initiation

Table 7. Reduction of tumor volume in xenograft mouse models implanted using human cancer cell lines after treatment with the combination of EGCG and anticancer drugs

Cancer cell line	Name of drugs	Tumor volume (% of control)				References
		Vehicle (control)	EGCG alone	Anticancer drugs alone	Combinations	
Prostate cancer cell lines						
PC-3ML	Paclitaxel	100	40.9[a]	44.3	0	Stearns and Wang, 2011
	Docetaxel	100	54.1[a]	42.4	0	Stearns and Wang, 2011
Average reduction of tumor volume (% of control)		100	73.5	66.3	29.7	Fujiki et al., 2015a

[a] 228 mg/kg EGCG

and tumor promotion, is a useful model for studying tumor promotion and cancer chemoprevention (Boutwell, 1977). We found that repeated applications of 5 mg EGCG before each treatment with 1 μg okadaic acid completely prevented tumor promotion in mouse skin initiated with DMBA (Yoshizawa et al., 1992). At that time, the dark basal keratinocytes in normal epidermal cells were assumed to be stem cells in two-stage carcinogenesis experiments in mouse skin (Slaga and Klein-Szanto, 1983). The epidermis is believed to contain two types of proliferating cells: stem cells and cells with a lower capacity for self-renewal and higher probability of undergoing terminal differentiation (transit amplifying cells) (Jones and Watt, 1993). The removal of the interfollicular epidermis from carcinogen-exposed mice, using an abrasion technique, reduced by half the number of papillomas, while the number of carcinomas remained the same in both abraded and unabraded mice (Morris et al., 2000). Stem cells are generally characterized by slow cycling, unlimited self-renewal, and multipotentiality, and they can commit to a variety of cell lineages that comprise the tissue of origin. Initiated label-retaining cells are clonogenic and differentiation-resistant, and hence a likely target for the mutagenic activity of chemical initiators and ultraviolet light (Gerdes and Yuspa, 2005). Mouse keratinocyte stem cells with $CD34^+$ and cytokeratin 15^+ (K15) are located only in the outer root sheath of a specific niche within the hair follicle defined as “the bulge,” which is thought to contain stem cells (Affara et al., 2006). Trempus's group reported that the back skin of CD34 knockout mice (CD34KO) initiated with DMBA and promoted with TPA failed to develop papillomas by week 20, compared with the wild-type mice. This suggests that CD34 is required for TPA-induced hair follicle stem cell activation and tumor formation in mice (Trempus et al., 2007). Blanpain's group showed that cancer stem cells (CSCs) of skin papillomas are localized in a perivascular niche, and that vascular endothelial growth factor (VEGF) affects skin tumor growth by promoting cancer stemness (the ability to self-renew and differentiate) and symmetric CSC division, leading to CSC expansion (Beck et al., 2011). Furthermore, they reported that SRY (sex determining region Y)-box 2 (Sox2) is the most upregulated transcription factor in the CSCs of squamous skin tumors in mice, and that Sox2 is absent in normal epidermis but begins to be expressed in the vast majority of mouse and human pre-neoplastic skin tumors (Boumahdi et al., 2014). The results indicated a strong relationship between cancer stem cells and expression of pluripotency-maintaining transcription factors.

EGCG and green tea extract inhibit the growth of human cancer cell lines in culture and in rodents (Fujiki et al., 2012; Okabe et al., 1999). Since inhibition of tumor promotion by EGCG is assumed to be strongly related to the non-toxic downregulation of CSCs, we gathered numerous reports from a search of PubMed and published our review article of the literature to provide a broad selection for the effects of EGCG on about 20 human CSCs enriched from cancer cell lines (Fujiki et al., 2017). In the next section, the expression of stemness markers in colorectal and nasopharyngeal CSCs will be introduced as examples, followed by the decrease or increase in stemness markers of human breast, lung and colorectal CSCs by EGCG.

HUMAN CSCS EXPRESS STEMNESS MARKERS DIFFERENTLY FROM THEIR PARENTAL CELLS

Human CSCs enriched from primary and secondary spheroids are capable of undergoing self-renewal. The quantitative differences in the levels of stemness markers between CSCs and parental cells were studied. The spheroid-derived CSCs, designated HCT116-SDCSCs, exhibit approximately 4.5-fold and 3.2-fold higher expression of stem cell markers, octamer-binding transcription factor 4 (*Oct4*) and Nanog homeobox protein (*Nanog*), respectively, than the parental cells (Toden et al., 2016). Oct4, Nanog, and Sox2 are transcription factors required for the maintenance of pluripotency by coordinated networks of transcription factors (Boumahdi et al., 2014; Kashyap et al., 2009; Sarkar and Hochedlinger, 2013). Moreover, the expression levels of the surface marker CD44 and self-renewal markers Notch homolog (Notch), B-lymphoma Moloney murine leukemia virus integration site 1 homologue (Bmi-1), CD133, and aldehyde dehydrogenase 1 (ALDH1) are higher in HCT116-SDCSCs than in parental cells (Table 8).

Compared with parental cells, human nasopharyngeal sphere-derived cells CSCs, designated TW01, express relatively high levels of the stem cell markers Sox2, Oct4, and Krüppel-like factor (Klf4), plus epithelial-mesenchymal transition (EMT) markers including Twist family BHLH transcription factor (Twist), Snail family transcriptional repressor (Snail), and vimentin, along with N-cadherin. However, decreased expression of *E-cadherin* in TW01 sphere-derived cells was detected (Table 8) (Lin et al., 2012).

Table 8. Human CSCs express stemness markers differentially

Cancers types and names of CSCs		Markers of increased expression	Markers of decreased expression	References
Colorectal cancer				
HCT116-SDCSCs	mRNAs:	*Oct4*, *Nanog*,		Toden et al., 2016
	Proteins:	CD44, Notch, Bmi-1, CD133, ALDH1		
Nasopharyngeal cancer				
TW01 sphere	mRNAs:	*Sox2, Oct4, KLf-4, Twist, Snail, Vimentin, N-cadherin*	*E-cadherin*	Lin et al., 2012

Table 9. EGCG decreases or increases the expression of stemness marker mRNAs and proteins in human CSCs.

Cancer types and names of CSCs	Inhibited expression of stemness markers (mRNAs and proteins)		References
Breast CSCs			
SUM-149 & SUM-190	mRNAs:	*CCND1, RHOC, BCL-XL*	Mineva et al., 2013
SUM-149	mRNAs:	*FN1, CDHI, Vimentin*	
Lung CSCs			
A549 & H1299	mRNAs:	*CD133,CD44, ALDH1A1, Nanog, Oct4*	Zhu et al., 2017
	Proteins:	CD133,CD44, ALDH1A1, Nanog, Oct4, PCNA, CyclinD1, Bcl2, β-Catenin, c-Myc	
	Increased	Bax, Caspase8, Cleaved Caspase-3 and -9	
Colorectal CSCs			
HCT116-5FUR	mRNAs:	*Oct4, Nanog*	Toden et al., 2016
& SW480-5FUR	Proteins:	Notch 1, cleaved-Notch 1, c-Myc, Bmi-1, Suz12, Ezh2	

EGCG DECREASES THE EXPRESSION OF MRNAS AND PROTEINS THAT SERVE AS STEMNESS MARKERS OF HUMAN CSCS

Breast CSCs: EGCG (40 μg/ml, 87.3 μM) inhibits the expression of genes that promote growth and contribute to the transformed phenotype and survival of SUM-190 spheres: In SUM-149 and SUM-190 cells, EGCG decreases the levels of mRNAs of the proliferation markers cyclin D1 (*CCND1*); ras homolog family member C (*RHOC*); and B-cell lymphoma-extra large (*BCL-XL*), which is a major antiapoptotic protein of the B-cell lymphoma 2 (Bcl2) family. EGCG also decreases ATP levels. In contrast, the levels of *Fibronectin 1* (*FN1*), *E-cadherin* (*CDHI*), and *Vimentin* decreased only in SUM-149 cells (Table 9). These results indicate that tumorsphere formation was inhibited by EGCG (Mineva et al., 2013).

The phenotypes of human estrogen receptor (ER)-negative MDA-MB-231 and MDA-MB-436 cells reflect tumors with a poor prognosis. In ER-negative breast cancer cell lines, ER-α36 is overexpressed and is associated with malignant growth (Zhang et al., 2011). EGCG (10 - 40 μM) inhibits its tumorsphere formation and down-regulates ER-α36 expression at 24 h, which is consistent with down-regulation of the epidermal growth factor receptor (EGFR). EGCG inhibits the growth of ER-negative human breast CSCs through down-regulation of ER-α36 expression, indicating that EGCG treatment will result in longer survival of patients with mammary cancers (Pan et al., 2016). The longer survival of patients who drink green tea was reported by Nakachi's group, as noted in the Introduction (Nakachi et al., 1998).

Lung CSCs: EGCG (0 - 100 μM) reduces the mRNA and protein levels of the lung CSC markers *CD133*, *CD44*, *ALDH1A1*, *Nanog,* and *Oct4* in CSC-A549 and CSC-H1299 cells, and also the protein levels of markers proliferating cell nuclear antigen (PCNA) and Cyclin D1 as well as that of Bcl2. In addition, EGCG reduces the protein levels of β-Catenin and v-Myc avian myelocytomatosis viral oncogene homolog (c-Myc). However, EGCG increases the levels of Bcl-2-associated X protein (Bax), Caspase 8, and cleaved Caspases-3 and -9 (Table 9). These results show that EGCG inhibits proliferation and induces apoptosis of lung CSCs (Zhu et al., 2017).

Colorectal CSCs: Compared with parental cells, 5-fluorouracil (5FU)-resistant (5FUR) CRC cells exhibit an increased ability to form spheroids, indicating the presence of a larger CSC population. EGCG (50 μM) inhibits tumorspheroid formation and the expression of the mRNAs of the stem cell markers *Oct4* and *Nanog*. Treatment of 5FUR CRCs with EGCG inhibits expression of all of the following: self-renewal markers that are components of the Notch homolog 1 (Notch) signaling pathway - Notch1, cleaved Notch1, and c-Myc - as well as that of the polycomb repressive complex subunits, Bmi-1, polycomb protein SUZ (Suz12), and enhancer of zeste homologue (Ezh2) (Table 9) (Toden et al., 2016).

The 30-year history of our studies is summarized in Fig. 1: Primary cancer prevention with green tea is for the general population, and it results in delayed cancer onset and reduced cancer incidence; secondary cancer prevention means early cancer diagnosis and treatment for the general population as well as cancer patients at clinics; and tertiary cancer prevention with the combination of green tea catechin and anticancer compounds is for cancer patients following cancer treatment (Fujiki et al., 2012; 2015a; 2015b; 2017).

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