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Research Article

The modulation of endothelial cell gene expression by green tea polyphenol-EGCG

Liping Liu[1], Chao-Qiang Lai[2], Lin Nie[1], Jose Ordovas[2], Michael Band[1], Leta Moser[1] and Mohsen Meydani[1]

[1] Vascular Biology Laboratory, JM USDA Human Nutrition Center on Aging at Tufts University, Boston, MA, USA
[2] Nutrition and Genomics Laboratory, JM USDA Human Nutrition Center on Aging at Tufts University, Boston, MA, USA

Human and animal studies have shown that green tea consumption is associated with a reduced risk of some cancers. This has been attributed to its polyphenol components, in particular (−)-epigallocatechin gallate (EGCG). In addition to be a cancer chemopreventive agent, EGCG inhibits angiogenesis, thus reducing tumor growth and metastasis. We tested EGCG modulation on the gene expression profile of endothelial cells stimulated by VEGF using Affymetrix microarrays. A total of 421 genes were up-regulated and 72 genes were down-regulated at the false discovery rate of 5% by VEGF, EGCG, and EGCG pretreatment followed by VEGF stimulation. The changes in the expression of several pivotal genes were validated by real-time PCR. Furthermore, we have identified two signaling pathways (Wnt and Id) involved in cell proliferation were inhibited by EGCG treatment, suggesting the negative regulation of EGCG on cell proliferation. Our results also indicate that the antiangiogenesis effect of EGCG is partially mediated through its broad inhibition on endothelial cell proliferation. Our data further support earlier observations that the anticancer effect of EGCG is mediated through changes in the expression of genes that are associated with cell proliferation.

Keywords: EGCG / Endothelial cell / MAPP analysis / Microarray / Proliferation

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1 Introduction

Epidemiological studies have shown that the consumption of green tea is associated with reduced risk of some cancers including gastric, esophagus, and pancreas [1–4]. Studies in animal models and *in vitro* cell culture system also suggest that green tea and green tea extract inhibit the development and progression of skin, lung, mammary gland, and gastrointestinal tumors [5, 6]. Although the protective mechanisms of green tea have not been fully elucidated, it has been proposed that green tea extract inhibits cell proliferation and causes cell apoptosis *in vitro* by modulating signal transduction [7]. The protective effect of green tea has been attributed to the biological activities of its polyphenol catechins content, in particular (−)-epigallocatechin gallate (EGCG), the major constituent in green tea extract, which has been shown to have significant antiproliferative and anticarcinogenic properties [8].

In addition to antiproliferative effects, green tea has been shown to inhibit tumor invasion and angiogenesis [9], which are the crucial steps for the growth and metastasis of all solid tumors. Angiogenesis, the formation of new blood vessels from the existing vessels, is involved in physiological processes such as wound healing as well as in pathological processes such as tumor growth and atherosclerosis [10, 11]. We and others have shown that EGCG inhibits tubular structure formation of endothelial cells in culture *via* modulation of vascular endothelial growth factor (VEGF) signaling, including phosphorylation of VEGF receptor and vascular endothelial (VE)-cadherin, disruption of VEGF-induced VE-cadherin/β-catenin complex, and inhibition of Akt phosphorylation and IL-8 production [12, 13]. It has been reported that the anticancer activity of EGCG is also associated with the inhibition of invasion by suppressing

Correspondence: Professor Mohsen Meydani, Vascular Biology Laboratory, JM USDA Human Nutrition Center on Aging at Tufts University, 711 Washington Street, Boston, MA 02111, USA
E-mail: mohsen.meydani@Tufts.edu
Fax: +1-617-556-3224

Abbreviations: CENPE, centromere protein E; **EGCG,** (−)-epigallocatechin gallate; **GO,** Gene Ontology; **VEGF,** vascular endothelial growth factor; **VE,** vascular endothelial; **HUVEC,** human umbilical vein endothelial cells

the activity of urokinase [14, 15] or the matrix metalloproteinases (MMPs) [16]. Thus, it appears that green tea EGCG may contribute to cancer prevention by reducing cell proliferation, tumor cell migration, and invasion, and by inhibiting angiogenesis. In order to gain further insights to the understanding of metabolic pathways that are affected by EGCG, we tested the effect of EGCG on genes expression in endothelial cells using Affymetrix microarrays system, which enabled us to analyze the signaling pathways modulated by EGCG based on changes in mRNA expressions.

2 Materials and methods

2.1 Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex (Walkersville, MD). Cells from passage 6 were grown in 100 mm Petri dishes. The cells were maintained with 2% fetal bovine serum (FBS) using certified EBM-2 medium supplemented with growth factor kit (EGM-^{TM}2 SingleQuots, Cambrex), which provides optimal condition for HUVEC proliferation. At 70% confluency, cells were supplemented with or without EGCG extracted from tea polyphenol fraction (LKT Laboratories, MN), 20 μM in DMSO for 24 h. DMSO final concentration in medium was 0.02%. Each treatment was performed as triplicate; 3 for control, 3 for EGCG, 3 for VEGF, 3 for EGCG + VEGF. We set up two sets of culturing dishes. The viability of the cells was around 95% during the treatment as measured by Trypan blue on one set of the dishes (data not shown). Since Abe and Sato [17] showed a very active gene expression profile following 30 min stimulation of HUVEC with VEGF, we therefore chose to stimulate HUVEC with 50 ng/mL VEGF for 30 min. Then the cells were washed and harvested for RNA extraction.

2.2 Measurement of cell proliferation

HUVECs were grown in 6-well plates up to 70% confluency. EGCG at concentrations of 5, 10, 20 μM and VEGF (final concentration 50 ng/mL) were added into the cells culture medium and cells were incubated for 24 h. The cells were trypsinized and cell suspension was prepared in 0.5 mL. Nine microliter was applied to hemocytometer and cell numbers *per* well were determined.

2.3 *In vitro* angiogenesis assay

Twenty-four-well plates were coated with ice-cold growth factor-reduced Matrigel (250 μL/well; BD, MA). It was allowed to polymerize at 37°C for 30 min. Thereafter, 1 mL of a suspension of HUVEC (2×10^5 cells/mL), which had been treated with DMSO or 20 μM EGCG in DMSO for up to 24 h, was seeded onto the Matrigel as described previously [18]. The cells were maintained in EBM-2 medium for 48 h in the presence or absence of VEGF (50 ng/mL). Tube formation was assessed after 48 h and quantified by determining the average length of total tubes and number of branching points in three randomly selected fields [18].

2.4 RNA extraction and preparation of biotin-labeled cRNA for genechip analysis

RNA was extracted from cells subjected to the different treatments using the RNeasy Mini Kit (Qiagen, Valencia CA) according to the manufacturer's protocol. Each experimental treatment was carried out in triplicate; 3 for control, 3 for EGCG, 3 for VEGF, 3 for EGCG + VEGF. Total 12 treated RNA went through hybridization. An aliquot of RNA from each culture dish was used on one RNA chip to do Microarray analysis. cDNA was synthesized from 5 μg total RNA by SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA). Then biotin-labeled cRNA was synthesized from cDNA with *in vitro* transcription. The cRNA samples were fragmented and hybridized to human U133A Array GeneChip (Affymetrix, Santa Clara, CA) in the 45°C oven overnight, at 60 rpm. Microarrays were washed and stained according to the protocol provided by the manufacturer. The arrays were scanned using Agilent confocal laser scanner (Affymetrix) and analyzed using Affymetrix 5.0 software (Affymetrix).

2.5 Data analysis of Affymetrix microarrays

Affymetrix Human U133A Array contains 22 283 human probe sets. Each gene on the array is represented by a probe set consisting of 11 perfect match (PM) and 11 mismatch (MM) probe pairs. The quantitative estimate of expression of each probe set is the Signal (Sig) metric. Sig is computed using the One-Step Tukey's Biweight Estimate, which gives the weighted mean of the log (PM-MM) intensities for each probe set (Affymetrix Microarray Suite, Version 5.0). All microarrays were scaled to the same target signal using the "All Probe Sets" scaling option. A detection call (present, marginal or absent) is also given for each probe set. Only those genes that, (i) had signal over 25 (the mean intensity of all probe sets that had "absent" call), and (ii) were called "Present" or "Marginal" in at least two of three replicates were included for statistical analysis. We used a Bayesian framework *t*-test (CyberT) [19] to identify genes that show differential expression among the control and treatments. The CyberT statistic models the SD as the function of signal intensity for each probe set using Bayesian framework. This approach was regarded as the most robust method to analyze Affymetrix microarray gene expression data [20]. Furthermore, we corrected *p*-values from the Bayesian framework *t*-test for multiple tests using a false-discovery rate of 5% (Q-values ≤ 0.05) criterion for significance [21].

2.6 Gene ontology and pathway analysis

We used the GenMapp 2.0 and MappFinder 2.0 software package (www.GenMapp.org) [22, 23] for Gene Ontology (GO) and pathway analysis. These resources contain most updated GO terms from GenMapp archives (www.GenMapp.org) and available pathways from KEGG (www.genome.jp/kegg) [24], as well as contribution of GenMapp users for humans. All the 8400 genes that were detectable in gene expression and their *Q*-values were imported into the programs, and linked to the GO hierarchy and pathway profiles. GO in GenMapp program allows all measured genes to be organized into hierarchies in three aspects of their biological functions: biological processes, cellular components, and molecular functions. Each hierarchy is structured in multiple levels of parent–child relationships between its terms. For each GO node, MappFinder calculated the percentage of the genes that met the user-defined criterion ($Q \leq 0.05$) among all the measured genes. We determined which GO terms and pathways (MAPP) were significantly over-represented by the set of significant genes by computing *Z*-scores. *Z*-scores were computed by subtracting the expected number of genes in a GO term (or MAPP), meeting the criterion from the observed number of genes, divided by the SD of the observed number of genes. A GO term or pathway with a *Z*-score greater than 1.96 ($p \leq 0.05$) was considered to be statistically significant and of potential biological importance. GO terms describing fewer than two genes that met the user-defined criteria were not considered here as they were either too specific or too general [23]. The filtered list of high *Z*-score GO terms was further annotated manually, to avoid redundant GO terms. When both a parent and a child GO term were presented in the list, the parent term was removed if its presence was due entirely to the genes meeting the criteria for the child term.

2.7 Validation of gene chip data by real time PCR

The aliquot of total RNA from microarray analysis was used to validate selected number of responsive genes. The first strand cDNA synthesis was performed with the SuperScript II reverse transcriptase (Invitrogen) by using 1 μg total RNA. The target mRNA levels were determined by TaqMan real-time RT-PCR using the ABI prism 7700 Sequence Detection System (Applied Biosystems, Foster city, CA) and the Ct method: the fold of suppression = $2^{Ct(control)-Ct(treatment)}$. The TaqMan probe consisted of an oligonucleotide with a 5′ reporter dye (FAM) and a downstream 3′ quencher dye (NFQ). The reactions were performed on plates using adhesive seals as covers. TaqMan RT-PCR master mix Reagent kit (Applied Biosystems) in a total volume of 20 μL consisted of 3 mM $MgCl_2$, 0.2 mM/each dNTP, 200 nM probe, primers, and 1.25 U Amplitaq Gold. The PCR was programmed as follows: initial denature at 95°C for 10 min followed by 95°C for 15 s, 60°C for 1 min, cycled 40 times. Each target was amplified in triplicates. The standard curve was constructed with serial dilutions of untreated endothelial cell RNA. A housekeeping gene β-actin was used as a reference for all of the samples. The primers were purchased from Applied Biosystems. They are premade primers and the catalog numbers are as follows: Hs00156507_m1(CENPE), Hs00357821_g1(ID1), Hs001 83740_m1(DKK1), Hs99999903_m1(beta-actin). Tryptophanyl-tRNA synthetase (TrpRS): forward primer: GCATGTAGGTCACCTCATTCCATTT, reverse primer: GACCAAGGGCACGTTAAATACATC, probe: CTGGAGCCACTTTGTG.

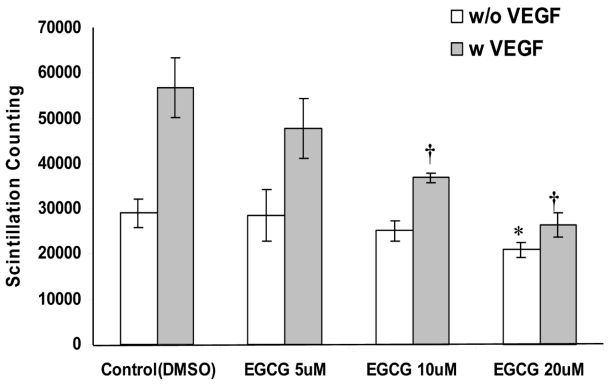

Figure 1. The inhibitory effect of doses of EGCG with and without VEGF-stimulation on HUVEC proliferation. HUVECs were cultured in 6-well plate until 70% confluent. Cells were incubated with or without 5, 10, and 20 μM EGCG in culture medium for 24 h, then stimulated with or with out 50 ng/mL VEGF for 24 h. The HUVEC proliferation rate was determined by thymidine incorporation assay. Data are mean ± SEM of duplicates *per* treatment. *$p < 0.05$ compared to control(DMSO) group. **$p < 0.05$ compared to DMSO plus VEGF treated group.

3 Results and discussion

3.1 Endothelial cell growth response to EGCG treatment

3.1.1 Cell proliferation

Cell proliferation modulated by EGCG preincubation was measured with and without VEGF stimulation. EGCG preincubation with and without VEGF stimulation dose-dependently inhibited HUVEC proliferation (Fig. 1).

3.1.2 *In vitro* angiogenesis

Endothelial cells growing on Matrigel coated plates showed tube formation, which represented the angiogenesis capacity of endothelial cells *in vivo*. The cells without VEGF stimulation, due to the presence of some growth factor in Matrigel, showed formation of tubes. VEGF stimulation increased tube formation by 15%. Both stimulated and unstimulated tube formations (mean length and numbers of

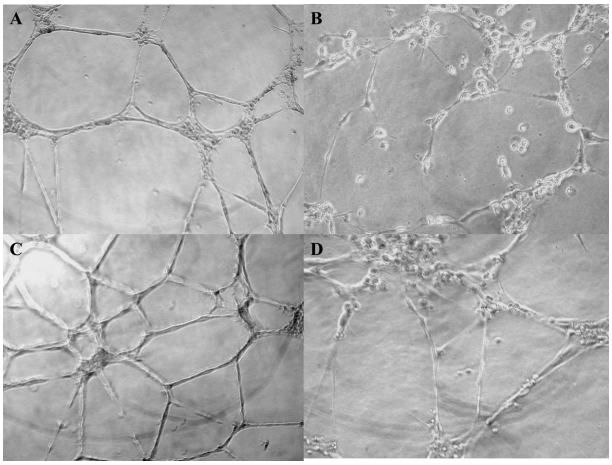

Figure 2. The inhibitory effect of EGCG on angiogenesis of HUVEC on Matrigel. HUVECs were cultured until 70% confluent. Cells were preincubated with or without 20 μM EGCG for 24 h. Then the cells were seeded on 24-well plates coated with growth factor reduced Matrigel and stimulated with or without 50 ng/mL VEGF for 48 h (see Section 2). Tube formations by endothelial cells on Matrigel were observed and photomicrographs of three random fields were taken after 48 h. (A) Control (DMSO); (B) 20 μM EGCG; (C) 50 ng/mL VEGF; (D) 20 μM EGCG + 50 ng/mL VEGF.

Table 1. Total numbers of genes that have been regulated by EGCG and VEGF

Treatment	Up-regulated		Down-regulated	
	[a] $p \leq 0.05$	[b] $Q \leq 0.05$	$p \leq 0.05$	$Q \leq 0.05$
EGCG *vs.* control	529	14	492	14
VEGF *vs.* control	710	291	638	51
EGCG + VEGF *vs.* VEGF	372	0	325	0
EGCG + VEGF *vs.* EGCG	425	116	257	7
Total	2036	421	1712	72

a) *p* values were calculated based on Bayesian framework *t*-test [19].
b) *Q*-value was estimated using *Q*-value software [21] at the false discovery rate of 5%.

branches of tubes in three random fields under the microscope) were inhibited by preincubation of endothelial cells with EGCG (20 μM) for 24 h (Figs. 2 and 3).

3.2 Gene expression responses to EGCG treatment in endothelial cells

Human U133A microarray contains 14 500 well-characterized human genes, of which the expressions of 8400 genes were detected from endothelial cells in our experiment. A total of 421 genes were up-regulated and 72 genes were down-regulated ($Q \leq 0.05$) by VEGF, EGCG, and EGCG pretreatment followed by VEGF stimulation (Table 1). We

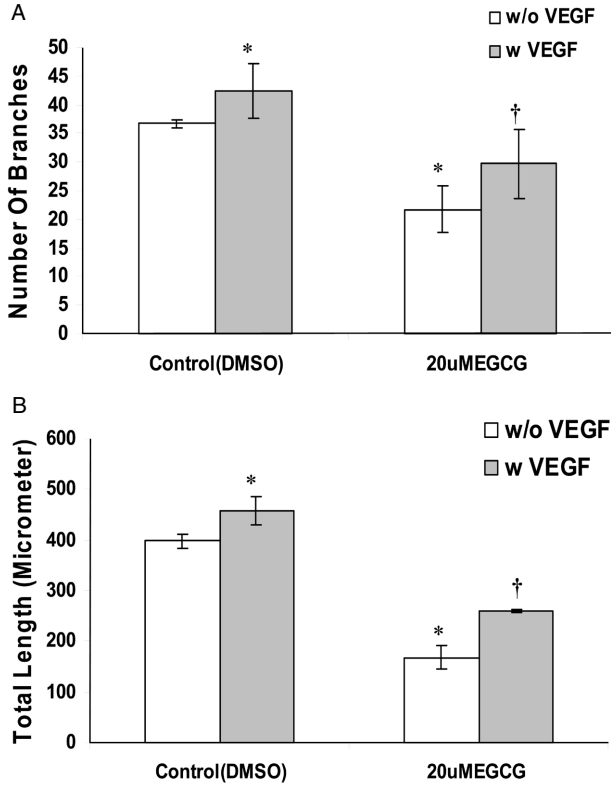

Figure 3. Quantification of total length of tubes and numbers of branches formed by HUVEC on Matrigel. HUVECs were cultured until 70% confluent. Cells were preincubated with or without 20 μM EGCG for 24 h. Then the cells were seeded on 24-well plates coated with growth factor reduced Matrigel and stimulated with or without 50 ng/mL VEGF for 48 h (see Section 2). Tube formations by endothelial cells on Matrigel were observed and photomicrographs of three random fields were taken after 48 h. The mean number of tube branches and length of tubes formed after 48 h in each field were counted. (A) The mean number of tube branches; (B) The mean number of tube length measured. Data are mean ± SEM, $n = 3$ *per* treatment. *$p < 0.05$ compared to DMSO control group, **$p < 0.05$ compared to DMSO plus VEGF treated group.

were specifically interested in the genes and functions that were affected by EGCG treatments, in particular, when cells were stimulated with VEGF.

3.2.1 The effects of VEGF on endothelial cells

VEGF at the concentrations of 50 ng/mL or more has been used to investigate angiogenesis in cell culture systems [25, 26]. In our previous studies, we have also used 50 ng/mL of VEGF *in vitro* to determine its mechanism of action on angiogenesis [27] To be consistent, we decided to use the same concentration of VEGF in the present study, which altered the expression of more than 350 genes in HUVECs within 30 min (Supporting Information Part 1). This effect of VEGF is mediated through specific binding to its receptors; VEGFR-1 (Flt-1) and VEG FR-2 (KDR/Fl k-1) on

Table 2. GO categories over-represented in probe sets significant for VEGF *vs.* control

GO ID	GO name[a)]	Number changed/number measured (% changed)	*Z*-Score	*p*-Value
Biological Process				
7250	Activation of NF-kappaB-inducing kinase	2/7 (28.6)	3.563	0.035
1525	Angiogenesis	4/25 (16.0)	3.870	0.001
6916	Antiapoptosis	10/52 (19.25)	5.486	0.002
19722	Calcium-mediated signaling	2/7 (28.6)	3.563	0.026
7050	Cell cycle arrest	7/42 (16.7)	4.584	<0.001
1709	Cell fate determination	2/5 (40)	7.637	<0.001
7267	Cell – cell signaling	17/92 (18.5)	5.691	<0.001
6935	Chemotaxis	9/37 (24.3)	7.571	<0.001
30574	Collagen catabolism	2/7 (28.6)	3.563	0.022
7253	Cytoplasmic sequestering of NF-kappaB	2/3 (66.7)	5.886	0.003
6959	Humoral immune response	2/13 (15.4)	2.306	0.029
188	Inactivation of MAPK activity	3/11 (27.3)	4.236	0.004
50930	Induction of positive chemotaxis	2/2 (100)	7.345	0.001
6954	Inflammatory response	6/61 (9.8)	3.656	0.003
7229	Integrin-mediated signaling pathway	4/26 (15.4)	3.249	0.013
7259	JAK-STAT cascade	2/13 (15.4)	2.871	0.034
30216	Keratinocyte differentiation	2/3 (66.7)	5.886	<0.001
7638	Mechanosensory behavior	2/3 (66.7)	5.886	0.003
7517	Muscle development	4/49 (8.2)	2.692	0.019
7422	Peripheral nervous system development	2/3 (66.7)	5.886	0.001
6470	Protein amino acid dephosphorylation	7/74 (9.5)	2.741	0.017
8277	Regulation of G-protein coupled receptor protein signaling pathway	3/12 (25.0)	4.001	0.007
9615	Response to virus	3/21 (14.3)	2.647	0.040
9611	Response to wounding	2/8 (25.0)	4.697	<0.001
1501	Skeletal development	5/33 (15.2)	2.732	0.013
6366	Transcription from RNA polymerase II promoter	10/115 (8.7)	4.890	<0.001
7179	Transforming growth factor beta receptor signaling pathway	3/14 (21.4)	2.871	0.031
1570	Vasculogenesis	2/4 (50.0)	5.002	0.011
Molecular function				
8009	Chemokine activity	6/11 (54.5)	9.112	<0.001
5125	Cytokine activity	6/31 (19.4)	8.820	<0.001
3677	DNA binding	19/506 (3.8)	5.322	<0.001
5355	Glucose transporter activity	2/4 (50)	5.002	0.007
8083	Growth factor activity	11/55 (20.0)	6.423	<0.001
5179	Hormone activity	5/17 (29.4)	5.346	<0.001
4879	Ligand-dependent nuclear receptor activity	2/5 (40.0)	4.308	0.002
17017	MAP kinase phosphatase activity	3/8 (37.5)	5.171	<0.001
8195	Phosphatidate phosphatase activity	2/3 (66.7)	5.886	0.002
8243	Plasminogen activator activity	2/5 (40.0)	4.388	0.008
19901	Protein kinase binding	3/11 (27.3)	2.546	0.044
3707	Steroid hormone receptor activity	5/24 (20.8)	4.431	0.001
3714	Transcription corepressor activity	9/58 (15.5)	4.920	<0.001
Cellular component				
5576	extracellular region	9/120 (7.5)	5.111	<0.001
5615	extracellular space	16/103 (15.5)	6.439	<0.001

a) GO terms listed met three criteria: (i) *Z* score ≥1.96, (ii) number genes with changed expression ≥ 2, and (iii) unique GO term only.

endothelial cells and downstream activating intracellular signaling pathways [28].

In accordance with previous works [17, 29, 30], our study has shown some typical regulatory effects of VEGF. GO term analysis identified the impact of VEGF on several biological processes including activation of NF-κB-inducing kinase, antiapoptosis, angiogenesis, and induction of positive chemotaxis (Table 2). Molecular functions such as chemokine and cytokine activities were also regulated by VEGF enrichment *in vitro* (Table 2). Similarly, MAPP pathway analysis has identified several pathways that have been regulated by VEGF stimulation, including apoptosis, adipo-

Table 3. Pathway analysis[a)]

MAPP name	Number changed/ number measured (% changed)	*Z*-Score	*p*-Value
EGCG *vs.* control			
Hs_Cholesterol_Biosynthesis	3/15 (20)	8.946	<0.001
Hs_Id_NetPath_5	2/43 (4.7)	3.117	0.039
Hs_Wnt_NetPath_8	2/77 (2.6)	2.016	0.108
VEGF *vs.* control			
Hs_Hypertrophy_model	7/19 (36.8)	5.230	<0.001
Hs_Adipogenesis	15/98 (15.3)	3.431	0.002
Hs_Cytokines_and_Inflammatory_Response_Biocarta	4/16 (25)	2.906	0.022
Hs_Apoptosis	10/73 (13.7)	2.388	0.028
Hs_Nuclear_Receptors	5/27 (18.5)	2.438	0.032
EGCG *vs.* EGCG + VEGF			
Hs_Hypertrophy_model	5/19 (26.3)	6.265	<0.001
Hs_Cytokines_and_Inflammatory_Response_Biocarta	4/16 (25.0)	5.424	0.001
Hs_Id_NetPath_5	4/43 (9.3)	2.626	0.038
Hs_Delta-Notch_NetPath_3	5/65 (7.7)	2.445	0.039
Hs_Blood_Clotting_Cascade	2/13 (15.4)	2.771	0.047
Hs_Smooth_muscle_contraction	6/101 (5.9)	1.978	0.054
Hs_Adipogenesis	6/98 (6.1)	2.059	0.060
Hs_Prostaglandin_synthesis_regulation	2/20 (10)	1.972	0.098

a) Pathway listed met two criteria: (i) *Z* score ≥1.96 and (ii) number genes with changed expression ≥2.

genesis, cytokine and inflammatory responses, and proliferation (Table 3).

3.2.2 The effects of EGCG on endothelial cells

Under EGCG treatments, there were 14 genes up-regulated and 14 genes down-regulated compared to the control group as calculated by both Bayesian *t*-test and *Q*-value correction (Table 1 and Supporting Information Part 2). To further elaborate the EGCG effect on modulation of cell proliferation, VEGF was added to stimulate cell growth (EGCG + VEGF treatment). Under this treatment, 116 genes were significantly up-regulated and 7 genes were down-regulated compared to the group with EGCG alone (Table 1 and Supporting Information Part 3). However, there was no differential gene expression when we compared the cells treated with EGCG + VEGF to the cells only treated with VEGF (Table 1).

Furthermore, based on GO analysis we found that EGCG exhibit strong regulatory effects on three biological processes: cholesterol biosynthesis, microtubule activities, and inhibition of cell proliferation (Table 4 and Supporting Information Part 2). As identified by MAPP finder (Table 3), 3 out of 15 genes (20%) in cholesterol biosynthesis pathway, have shown differential expressions: Isopentenyl-diphosphate delta isomerase, dehydrocholesterol reductase, and sterol-C4-methyl oxidase-like genes (please see the pathway cholesterol biosynthesis at this site: (http://www.wikipathways.org/index.php/Pathway:Human:Cholesterol_Biosynthesis). The expressions of all these three genes were increased by EGCG treatment (Table 4 and Supporting Information Part 2). The increase of dehydrocholesterol reductase and sterol-C4-methyl oxidase-like genes would likely contribute to the synthesis of cholesterol. The significance of this effect of EGCG in endothelial cells warrants further investigation.

3.2.3 Negative regulation of EGCG on endothelial cell proliferation and angiogenesis

The negative regulation of EGCG on endothelial cell proliferation in this study (Table 4) appears to have a profound biological significance. TGFβ inducible early growth responses gene (TIEG) and tryptophanyl-tRNA synthetase (WARS), were 2 out of 78 genes (2.6%) that are involved in cell proliferation were regulated by EGCG in the current study (Table 4 and Supporting Information Part 2). TIEG belongs to a family of transcription factors with antiproliferative and apoptosis-inducing functions [31, 32] However, we found that this gene was down-regulated by EGCG treatment (Supporting Information Part 2), whereas with VEGF stimulation, TIEG was up-regulated by EGCG compared to the control (Supporting Information Part 3). Thus, the down-regulation of TIEG represents the anti-VEGF function of EGCG and might be tissue specific. WARS is an enzyme that catalyzes the first step of protein synthesis, and is reported to have antiangiogenesis function in addition to the catalyzing function [33, 34]. It binds to intercellular junctions of endothelial cells to form a complex with VE-cadherin which then inhibits VEGF-induced ERK activation and endothelial cell migration [35]. The up-regulation of WARS by EGCG and inhibition of angiogenesis may counteract down-regulation of TIEG as noted with

Table 4. GO categories over-represented in probe sets significant for EGCG *vs.* control

GO ID	GO name[a)]	Number changed/number measured (% changed)	*Z*-Score	*p*-Value
Biological process				
6695	Cholesterol biosynthesis	3/17 (17.6)	11.187	<0.001
7018	Microtubule-based movement	2/40 (5.0)	4.751	0.014
8285	Negative regulation of cell proliferation	2/78 (2.6)	3.221	0.036
Molecular function				
3777	Microtubule motor activity	2/64 (3.1)	3.276	0.038
16491	Oxidoreductase activity	2/33 (6.1)	5.360	0.007
16853	Isomerase activity	5/251 (2.0)	3.566	0.004
Cellular component				
5783	ER	4/194 (2.1)	3.088	0.017
5624	Membrane fraction	3/201 (1.5)	3.332	0.017
5875	Microtubule associated complex	2/27 (7.4)	4.448	0.010

a) GO terms listed met three criteria: (i) *Z* score ≥1.96, (ii) number genes with changed expression ≥2, and (3) unique GO term only.

EGCG treatment (Supporting Information Part 3). In terms of biological outcome, our finding is in accordance with the previous observations showing the suppressive effect of EGCG on the proliferation of different cell types. For examples, studies have shown EGCG inhibited the growth of human epidermoid carcinoma cells [36] hepatic stellate cells [37], human bronchial epithelial 21BES cells [38] and human cervical cancer cells [39]. One study reported that EGCG broadly decreased the expression of genes related with prostate cancer cells proliferation [40]. And another study reported EGCG modulated early response genes in bronchial epithelial 21BES cells followed by activation of genes with a variety of cellular functions such as growth inhibition and the activation of apoptosis that are downstream targets of early response genes [38].

Two pathways related to cell proliferation were modified by EGCG treatment in our study: Wnt signaling pathway and Id pathway. Wnts are a family of signaling proteins secreted from various cells, which have a major impact on embryonic development, tumor progression, and stem cell differentiation [41] (please see the Wnt signaling pathway at: (http://www.wikipathways.org/index.php/Pathway:Human:Wnt_-signaling). Studies have shown that Wnt pathway is activated in endothelial cells *in vitro*. The activated endothelial cells express multiple ligands, receptors, and secretory modulators, which may play roles in angiogenesis [42]. Wnt signaling also induces proliferation and survival of endothelial cells [43, 44]. This pathway is triggered by Wnt binding to its cell membrane receptors, which is composed of ten transmembrane proteins [45]. The signal is then transduced through several cascades of cytoplasmic proteins to nucleus *via* the translocation of the complex formed by β-catenin and LEF/TCF. The complex exerts transcriptional activity [46] (I agree to get rid of this part). In the current study, 2 out of 77 genes in Wnt pathway were regulated by EGCG treatment. Dickkopf homolog 1 (Dkk1) is a soluble antagonist of Wnt pathway and has a high affinity ligand to LRP5, which is a key molecule in Wnt pathway functioning as a coreceptor for Wnt binding to the cell membrane [47]. The induction of Dkk1 expression by EGCG in our study (Table 4 and Supporting Information Part 2) is an indication of Wnt signaling pathway inhibition at the mRNA level.

Centromere protein E (CENPE) is a kinesin-like motor protein that accumulates in the G2 phase of the cell cycle. CENPE is proposed to be one of the motors responsible for mammalian chromosome movement and/or spindle elongation, and involved in Wnt signaling pathway. Its reduction by EGCG (Table 4 and Supporting Information Part 2) further indicates the inhibition of cell proliferation by EGCG. In addition, inhibitors of differentiation (Id) or DNA binding proteins are important for cellular proliferation and differentiation in a variety of cell types through regulation of gene expression (please see Id pathway at this site: (http://www.wikipathways.org/index.php/Pathway:Human:Id_-NetPath_5). Id-1 belongs to a group of helix-loop-helix proteins that lack DNA binding domain. Id-1 inhibits DNA binding activity of basic helix-loop-helix (bHLH) by forming nonfunctional heterodimers with it to inhibit cell differentiation and promote proliferation [48]. Studies have shown that Id-1 induces proliferation in prostate cancer cells, hepatocellular carcinoma cells, and nasopharyngeal carcinoma cells [48]. Its down-regulation by EGCG (Table 4 and Supporting Information Part 2) is likely to play a role in the inhibition of tumorigenesis and progression. Hairy and enhancer of split 1 (HES1) is another HLH-type regulator of cell proliferation [49, 50]. The down-regulation of this gene by EGCG (Table 4 and Supporting Information Part 2) probably results in the inhibition of proliferation. The inhibition of genes involved in cell proliferation by EGCG was also confirmed by the dose-dependent inhibition of HUVEC

Table 5. GO categories over-represented in probe sets significant for EGCG + VEGF *vs.* EGCG

GO ID	GO name[a)]	Number changed/number measured (% changed)	*Z*-Score	*p*-Value
Biological process				
1525	Angiogenesis	3/25 (12.0)	4.275	0.007
6916	Antiapoptosis	5/52 (9.6)	4.902	<0.001
6874	Calcium ion homeostasis	2/5 (40.0)	4.349	0.016
19722	Calcium-mediated signaling	2/7 (28.6)	6.448	0.002
7050	Cell cycle arrest	3/42 (7.1)	3.405	0.018
6928	Cell motility	2/59 (3.4)	3.749	0.006
7267	Cell – cell signaling	12/92 (13.0)	7.085	<0.001
6935	Chemotaxis	7/37 (18.9)	10.911	<0.001
7186	G-protein coupled receptor protein signaling pathway	4/88 (4.5)	3.840	0.003
6959	Humoral immune response	2/13 (15.4)	4.092	0.003
50930	Induction of positive chemotaxis	2/2 (100)	12.459	<0.001
6954	Inflammatory response	6/61 (9.8)	7.760	<0.001
7229	Integrin-mediated signaling pathway	2/26 (7.6)	2.927	0.048
30216	Keratinocyte differentiation	2/3 (66.7)	10.108	0.001
7517	Muscle development	2/49 (4.1)	2.588	0.039
8285	Negative regulation of cell proliferation	7/78 (9.0)	6.054	<0.001
122	Negative regulation of transcription from RNA polymerase II promoter	3/39 (7.7)	3.525	0.016
7399	Neurogenesis	8/107 (7.5)	5.878	<0.001
9887	Organogenesis	3/33 (9.1)	8.144	<0.001
8284	Positive regulation of cell proliferation	6/55 (10.9)	5.996	0.001
8217	Regulation of blood pressure	2/8 (25.0)	5.992	0.006
8277	Regulation of G-protein coupled receptor protein signaling pathway	2/12 (16.7)	4.762	0.008
9618	Response to pathogenic bacteria	2/7 (28.6)	5.288	0.012
1501	Skeletal development	3/33 (9.1)	3.240	0.034
6366	Transcription from RNA polymerase II promoter	4/115 (3.5)	4.025	<0.001
Molecular function				
8009	Chemokine activity	6/11 (54.5)	15.778	<0.001
5125	Cytokine activity	3/31 (9.7)	12.01	<0.001
3677	DNA binding	10/506 (2.0)	5.311	<0.001
8083	Growth factor activity	7/55 (12.7)	7.904	<0.001
8201	Heparin binding	3/29 (10.3)	4.369	0.008
5179	Hormone activity	2/17 (11.8)	3.604	0.022
17017	MAP kinase phosphatase activity	3/8 (37.5)	9.148	0.001
8243	Plasminogen activator activity	2/5 (40.0)	7.729	0.002
3707	Steroid hormone receptor activity	3/24 (12.5)	4.795	0.001
3714	Transcription corepressor activity	5/58 (8.6)	5.015	<0.001
Cellular component				
5576	Extracellular region	4/121 (3.3)	6.595	<0.001
5615	Extracellular space	11/103 (10.7)	8.432	<0.001

a) GO terms listed met three criteria: (i) *Z* score ≥1.96, (ii) number genes with changed expression ≥2, and (iii) unique GO term only.

treated with EGCG or stimulated with VEGF following EGCG supplement (Fig. 1). Our findings on the EGCG suppressive effect on VE cells proliferation and those reported on cancer cells suggest that the overall antiproliferative action of EGCG might be one important mechanism by which this polyphenols of green tea contributes to the prevention of angiogenesis and cancer progression.

We and others have shown that EGCG inhibits angiogenesis [12, 51]. While endothelial cell proliferation is one of the stages of angiogenesis, other molecular mechanisms are also involved in this process. In the present study, we have identified relevant molecules involved in angiogenesis as affected by EGCG. GO term analysis discovered that EGCG has profound influence on intracellular microtubule components and activities (Tables 4 and 5), which may play a role in the inhibition of cell migration and angiogenesis as well. Further studies are needed to determine EGCG modulation at protein levels in conjunction with biological functions presented here: the inhibition of cell proliferation and angiogenesis.

3.2.4 The effects of EGCG on endothelial cells stimulated with VEGF

When EGCG-pretreated HUVECs were stimulated with VEGF, a more robust effect of EGCG on biological functions and signal transduction pathways was observed compared to the HUVECs treated with EGCG alone (Tables 5 and Supporting Information Part 3). However, despite of EGCG inhibition of cell growth and angiogenesis (Figs. 1 – 3), we did not see any change with EGCG pretreatment in the most well-known genes that are involved in the classic angiogenesis-signaling pathway such as PI3 kinase and dual specificity phosphatase (PTEN). These observations were in concurrence with the previous report demonstrating that EGCG had no effect on the expression of PI3 kinase and PTEN [40]. Our previous study also showed that EGCG inhibits angiogenesis by preventing phosphorylation of several enzymes involved in angiogenesis signal transduction pathways [27]. EGCG, through its direct binding to receptors and enzymes [52, 53], or through its metal chelating activity [54], may regulate some enzymes and cell surface receptors, which are dependent on divalent cation for their activation [8, 54]. Furthermore, we have shown that EGCG inhibits angiogenesis through the disruption of VEGF-induced VE-cadherin/VEGF receptors/β-catenin/PI3-kinase complex formation [27] in endothelial cells without affecting gene expressions of the enzymes. Therefore, the antiangiogenesis effect of EGCG is mainly due to the inhibition of the activity of enzymes that are involved in signaling pathways that govern vessel formation and maturation.

When HUVECs were incubated with EGCG for 24 h, then stimulated with VEGF for 30 min, there was no significant difference on gene expression compared to the cells that were stimulated with VEGF only. However, in comparison to EGCG treatment, 123 genes showed differential expressions. This differential effect can be attributed to the strong mitogenic effect of VEGF. VEGF has been shown to exert a dynamic modulatory effect on endothelial cell gene expressions associated with cell proliferation, migration, and angiogenesis [17].

We have validated our findings by determining mRNA levels of several selected genes using quantitative real-time RT-PCR (QPCR) (Fig. 4). Our QPCR results showed that EGCG treatment has inhibitory effects on Id1 and CENPE gene expression levels and induced the expressions of DKK1 and WARS. The fold of changes detected by QPCR was consistent with Microarray analysis.

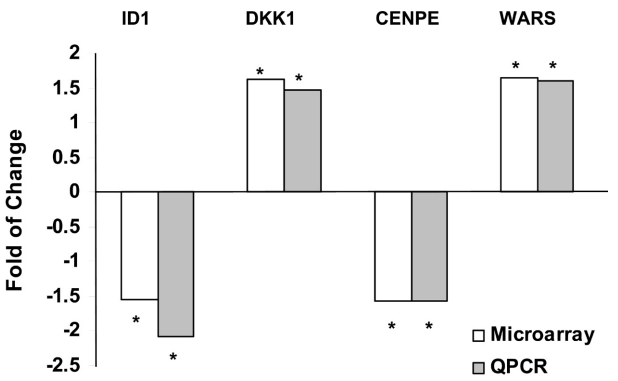

Figure 4. QPCR validation of selected differentially expressed genes by EGCG treatment. HUVECs were cultured in 100 mm Petri dishes until 70% confluent. Cells were incubated with or without 20 μM EGCG for 24 h. Total RNA was extracted for both microarray and quantitative real-time PCR analysis (see Section 2). $n = 5$ *per* treated group. $^*p < 0.05$ compared to control (DMSO) group. Abbreviations: ID1, inhibitors of differentiation protein 1; DKK1, Dickkopf (Xenopus laevis) homolog 1; CENPE, centromere protein E; WARS, tryptophanyl-tRNA synthetase; *Y*-axis, fold of change; EGCG treated group compared to control.

4 Concluding remarks

This study demonstrates the modulation of endothelial cell gene expression by EGCG when the cells were challenged with angiogenic growth factor VEGF. We have found that a combination of various cellular regulatory components is required for EGCG to exert its antiangiogenic effect. A selective number of genes modulated by EGCG were validated by real time PCR. It should be noted that *in vitro* levels of VEGF and EGCG applied in this study are comparable to those used by other investigators [39, 40, 55]. The antiangiogenic activity of EGCG on endothelial cells might be through inhibition of cell proliferation and cell migration. Future work is needed to elucidate the biological function of those specific genes by employing transgenic and molecular biology approaches. These EGCG-responsive genes may provide key insights for identifying the mechanisms of other polyphenolic compounds that might have cancer prevention properties.

In the present study, 20 μM EGCG has shown to inhibit HUVEC proliferation. Others used 10 – 25 μM to investigate inhibitory effect of EGCG on endothelial cells and higher than 25 μM on cancer cell proliferation studies [56]. Studies have shown serum level of EGCG reaches 5 μM with daily moderate consumption of green tea and other beverages containing green tea extract that are currently available in the market, but not substantially increases with higher level consumption [57, 58]. It appears that EGCG not only has nutritional impact on human health, but it may have pharmacological applications for prevention and treatment of abnormal cell growth. Further studies are warranted to explore this aspect, which may provide insights for new drug development.

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5 References

[1] Kono, S., Ikeda, M., Tokudome, S., Kuratsune, M., A case-control study of gastric cancer and diet in northern Kyushu, Japan, *Jpn. J. Cancer Res*. 1988, *79*, 1067 – 1074.

[2] Gao, Y. T., McLaughlin, J. K., Blot, W. J., Ji, B. T., *et al*., Reduced risk of esophageal cancer associated with green tea consumption, *J. Natl. Cancer Inst*. 1994, *86*, 855 – 858.

[3] Setiawan, V. W., Zhang, Z. F., Yu, G. P., Lu, Q. Y., *et al*., Protective effect of green tea on the risks of chronic gastritis and stomach cancer, *Int. J. Cancer* 2001, *92*, 600 – 604.

[4] Bushman, J. L., Green tea and cancer in humans: A review of the literature, *Nutr. Cancer* 1998, *31*, 151 – 159.

[5] Rogers, A. E., Hafer, L. J., Iskander, Y. S., Yang, S., Black tea and mammary gland carcinogenesis by 7,12-dimethylben[a]-anthracene in rats fed control or high fat diets, *Carcinogenesis* 1998, *19*, 1269 – 1273.

[6] Lyn-Cook, B. D., Rogers, T., Yan, Y., Blann, E. B., *et al*., Chemopreventive effects of tea extracts and various components on human pancreatic and prostate tumor cells in vitro, *Nutr. Cancer* 1999, *35*, 80 – 86.

[7] Park, A. M., Dong, Z., Signal transduction pathways: Targets for green and black tea polyphenols, *J. Biochem. Mol. Biol.* 2003, *36*, 66 – 77.

[8] Jung, Y. D., Ellis, L. M., Inhibition of tumour invasion and angiogenesis by epigallocatechin gallate (EGCG), a major component of green tea, *Int. J. Exp. Pathol*. 2001, *82*, 309 – 316.

[9] Cao, Y., Cao, R., Angiogenesis inhibited by drinking tea, *Nature* 1999, *398*, 381.

[10] Folkman, J., Angiogenesis in cancer, vascular, rheumatoid and other disease, *Nat. Med.* 1995, *1*, 27 – 31.

[11] Carmeliet, P., Jain, R. K., Angiogenesis in cancer and other diseases, *Nature* 2000, *407*, 249 – 257.

[12] Tang, F. Y., Meydani, M., Green tea catechins and vitamin E inhibit angiogenesis of human microvascular endothelial cells through suppression of IL-8 production, *Nutr. Cancer* 2001, *41*, 119 – 125.

[13] Tang, F. Y., Nguyen, N., Meydani, M., Green tea catechins inhibit VEGF-induced angiogenesis in vitro through suppression of VE-cadherin phosphorylation and inactivation of Akt molecule, *Int. J. Cancer* 2003, *106*, 871 – 878.

[14] Jankun, J., Selman, S. H., Swiercz, R., Skrzypczak-Jankun, E., Why drinking green tea could prevent cancer, *Nature* 1997, *387*, 561.

[15] Jankun, J., Keck, R. W., Skrzypczak-Jankun, E., Swiercz, R., Inhibitors of urokinase reduce size of prostate cancer xenografts in severe combined immunodeficient mice, *Cancer Res*. 1997, *57*, 559 – 563.

[16] Garbisa, S., Sartor, L., Biggin, S., Salvato, B. *et al*., Tumor gelatinases and invasion inhibited by the green tea flavanol epigallocatechin-3-gallate, *Cancer* 2001, *91*, 822 – 832.

[17] Abe, M., Sato, Y., cDNA microarray analysis of the gene expression profile of VEGF-activated human umbilical vein endothelial cells, *Angiogenesis* 2001, *4*, 289 – 298.

[18] Grant, D. S., Lelkes, P. I., Fukuda, K., Kleinman, H. K., Intracellular mechanisms involved in basement membrane induced blood vessel differentiation in vitro, *In Vitro Cell Dev. Biol*. 1991, *27A*, 327 – 336.

[19] Baldi, P., Long, A. D., A Bayesian framework for the analysis of microarray expression data: Regularized t-test and statistical inferences of gene changes, *Bioinformatics* 2001, *17*, 509 – 519.

[20] Choe, S. E., Boutros, M., Michelson, A. M., Church, G. M., Halfon, M. S., Preferred analysis methods for Affymetrix GeneChips revealed by a wholly defined control dataset, *Genome Biol*. 2005, *6*, R16.

[21] Storey, J. D., Tibshirani, R., Statistical significance for genomewide studies, *Proc. Natl. Acad. Sci. USA* 2003, *100*, 9440 – 9445.

[22] Dahlquist, K. D., Salomonis, N., Vranizan, K., Lawlor, S. C., Conklin, B. R., GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways, *Nat. Genet.* 2002, *31*, 19 – 20.

[23] Doniger, S. W., Salomonis, N., Dahlquist, K. D., Vranizan, K. *et al*., MAPPFinder: Using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data, *Genome Biol*. 2003, *4*, R7.

[24] Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y., Hattori, M., The KEGG resource for deciphering the genome, *Nucleic Acids Res*. 2004, *32*, D277 – D280.

[25] Osada, R., Horiuchi, A., Kikuchi, N., Ohira, S., *et al*., Expression of semaphorins, vascular endothelial growth factor, and their common receptor neuropilins and alleic loss of semaphorin locus in epithelial ovarian neoplasms: Increased ratio of vascular endothelial growth factor to semaphorin is a poor prognostic factor in ovarian carcinomas, *Hum. Pathol*. 2006, *37*, 1414 – 1425.

[26] Cai, J., Jiang, W. G., Ahmed, A., Boulton, M., Vascular endothelial growth factor-induced endothelial cell proliferation is regulated by interaction between VEGFR-2, SH-PTP1 and eNOS, *Microvasc. Res*. 2006, *71*, 20 – 31.

[27] Rodriguez, S. K., Guo, W., Liu, L., Band, M. A., *et al*., Green tea catechin, epigallocatechin-3-gallate, inhibits vascular endothelial growth factor angiogenic signaling by disrupting the formation of a receptor complex, *Int. J. Cancer* 2006, *118*, 1635 – 1644.

[28] Millauer, B., Wizigmann-Voos, S., Schnurch, H., Martinez, R., *et al*., High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis, *Cell* 1993, *72*, 835 – 846.

[29] Gerritsen, M. E., Tomlinson, J. E., Zlot, C., Ziman, M., Hwang, S., Using gene expression profiling to identify the molecular basis of the synergistic actions of hepatocyte growth factor and vascular endothelial growth factor in human endothelial cells, *Br. J. Pharmacol.* 2003, *140*, 595 – 610.

[30] Kallmann, B. A., Wagner, S., Hummel, V., Buttmann, M., *et al*., Characteristic gene expression profile of primary human cerebral endothelial cells, *FASEP J.* 2002, *16*, 589 – 591.

[31] Tachibana, I., Imoto, M., Adjei, P. N., Gores, G. J., *et al*., Overexpression of the TGFbeta-regulated zinc finger encoding gene, TIEG, induces apoptosis in pancreatic epithelial cells, *J. Clin. Invest.* 1997, *99*, 2365 – 2374.

[32] Ribeiro, A., Bronk, S. F., Roberts, P. J., Urrutia, R., Gores, G. J., The transforming growth factor beta(1)-inducible transcription factor TIEG1, mediates apoptosis through oxidative stress, *Hepatology* 1999, *30*, 1490 – 1497.

[33] Wakasugi, K., Slike, B. M., Hood, J., Otani, A., *et al*., A human aminoacyl-tRNA synthetase as a regulator of angiogenesis, *Proc. Natl. Acad. Sci. USA* 2002, *99*, 173 – 177.

[34] Otani, A., Slike, B. M., Dorrell, M. I., Hood, J., *et al*., A fragment of human TrpRS as a potent antagonist of ocular angiogenesis, *Proc. Natl. Acad. Sci. USA* 2002, *99*, 178 – 183.

[35] Tzima, E., Reader, J. S., Irani-Tehrani, M., Ewalt, K. L., *et al*., VE-cadherin links tRNA synthetase cytokine to anti-angiogenic function, *J. Biol. Chem*. 2005, *280*, 2405 – 2408.

[36] Liang, Y. C., Lin-shiau, S. Y., Chen, C. F., Lin, J. K., Suppression of extracellular signals and cell proliferation through EGF receptor binding by (–)-epigallocatechin gallate in human A431 epidermoid carcinoma cells, *J. Cell. Biochem*. 1997, *67*, 55 – 65.

[37] Chen, A., Zhang, L., Xu, J., Tang, J., The antioxidant (–)-epigallocatechin-3-gallate inhibits activated hepatic stellate cell growth and suppresses acetaldehyde-induced gene expression, *Biochem. J*. 2002, *368*, 695 – 704.

[38] Vittal, R., Selvanayagam, Z. E., Sun, Y., Hong, J., *et al*., Gene expression changes induced by green tea polyphenol (–)-epigallocatechin-3-gallate in human bronchial epithelial 21BES cells analyzed by DNA microarray, *Mol. Cancer Ther*. 2004, *3*, 1091 – 1099.

[39] Ahn, W. S., Huh, S. W., Bae, S. M., Lee, I. P., *et al*., A major constituent of green tea, EGCG, inhibits the growth of a human cervical cancer cell line, CaSki cells, through apoptosis, G(1) arrest, and regulation of gene expression, *DNA Cell Biol*. 2003, *22*, 217 – 224.

[40] Wang, S. I., Mukhtar, H., Gene expression profile in human prostate LNCaP cancer cells by (–)-epigallocatechin-3-gallate, *Cancer Lett.* 2002, *182*, 43 – 51.

[41] Eisenberg, L. M., Eisenberg, C. A., Wnt signal transduction and the formation of the myocardium, *Dev Biol*. 2006, *293*, 305 – 315.

[42] Goodwin, A. M., Sullivan, K. M., D'Amore, P. A., Cultured endothelial cells display endogenous activation of the canonical Wnt signaling pathway and express multiple ligands, receptors, and secreted modulators of Wnt signaling, *Dev. Dyn*. 2006, *235*, 3110 – 3120.

[43] Masckauchan, T. N., Agalliu, D., Vorontchikhina, M., Ahn, A. *et al*., Wnt5a signaling induces proliferation and survival of endothelial cells in vitro and expression of MMP-1 and Tie-2, *Mol. Biol. Cell* 2006, *17*, 5163 – 5172.

[44] Masckauchan, T. N., Kitajewski, J., Wnt/Frizzled signaling in the vasculature: New angiogenic factors in sight, *Physiology (Bethesda)* 2006, *21*, 181 – 188.

[45] Huang, H. C., Klein, P. S., The Frizzled family: Receptors for multiple signal transduction pathways, *Genome Biol*. 2004, *5*, 234.

[46] Logan, C. Y., Nusse, R., The Wnt signaling pathway in development and disease, *Annu. Rev. Cell Dev. Biol*. 2004, *20*, 781 – 810.

[47] He, X., Semenov, M., Tamai, K., Zeng, X., LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: Arrows point the way, *Development* 2004, *131*, 1663 – 1677.

[48] Hui, C. M., Cheung, P. Y., Ling, M. T., Tsao, S. W., *et al*., Id-1 promotes proliferation of p53-deficient esophageal cancer cells, *Int. J. Cancer* 2006, *119*, 508 – 514.

[49] Bae, S., Bessho, Y., Hojo, M., Kageyama, R., The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation, *Development* 2000, *127*, 2933 – 2943.

[50] Ishiko, E., Matsumura, I., Ezoe, S., Gale, K., *et al*., Notch signals inhibit the development of erythroid/megakaryocytic cells by suppressing GATA-1 activity through the induction of HES1, *J. Biol. Chem*. 2005, *280*, 4929 – 4939.

[51] Kondo, T., Ohta, T., Igura, K., Hara, Y., Kaji, K., Tea catechins inhibit angiogenesis in vitro, measured by human endothelial cell growth, migration and tube formation, through inhibition of VEGF receptor binding, *Cancer Lett*. 2002, *180*, 139 – 144.

[52] Tachibana, H., Koga, K., Fujimura, Y., Yamada, K., A receptor for green tea polyphenol EGCG, *Nat. Struct. Mol. Biol*. 2004, *11*, 380 – 381.

[53] Haslam, E., Natural polyphenols (vegetable tannins) as drugs: Possible modes of action, *J. Nat. Prod.* 1996, *59*, 205 – 215.

[54] Maeda-Yamamoto, M., Kawahara, H., Tahara, N., Tsuji, K., *et al*., Effects of tea polyphenols on the invasion and matrix metalloproteinases activities of human fibrosarcoma HT1080 cells, *J. Agric. Food Chem*. 1999, *47*, 2350 – 2354.

[55] Okabe, S., Fujimoto, N., Sueoka, N., Suganuma, M., Fujiki, H., Modulation of gene expression by (–)-epigallocatechin gallate in PC-9 cells using a cDNA expression array, *Biol. Pharm. Bull*. 2001, *24*, 883 – 886.

[56] Fassina, G., Vene, R., Morini, M., Minghelli, S., *et al*., Mechanisms of inhibition of tumor angiogenesis and vascular tumor growth by epigallocatechin-3-gallate, *Clin. Cancer Res*. 2004, *10*, 4865 – 4873.

[57] Scalbert, A., Williamson, G., Dietary intake and bioavailability of polyphenols, *J. Nutr*. 2000, *130*, 2073S – 2085S.

[58] Chow, H. H., Cai, Y., Hakim, I. A., Crowell, J. A., *et al*., Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals, *Clin. Cancer Res*. 2003, *9*, 3312 – 3319.

