# EGCG Suppresses Melanoma Tumor Angiogenesis and Growth without Affecting Angiogenesis and VEGF Expression in the Heart and Skeletal Muscles in Mice

Kevan B. Tucker[1,2], Kristina L. Makey[1,2], Edmund Chinchar[1,2], Min Huang[2], Natale Sheehan[3], Srinivasan Vijayakumar[1] and Jian-Wei Gu[1,2,*]

*[1]Cancer Institute; [2]Department of Physiology & Biophysics; [3]Department of Medicine University of Mississippi Medical Center, Jackson, Mississippi 39216, USA*

**Abstract:** Melanoma is a highly malignant cancer with a potent capacity to metastasize distantly and has a higher mortality. There is no effective therapy for high risk melanoma patients to prevent relapse or distant metastasis. Therefore effective chemoprevention strategies are needed. The present study mainly evaluates the effects of EGCG on melanoma angiogenesis, growth, and capillary density (CD) in the heart and skeletal muscles of mice. 5 x 10^5 B16F10 cells were inoculated into the right proximal dorsal of the back in the eight week old male mice (n=12). Then, 6 mice received EGCG at 50–100 mg/kg/d in drinking water for 4 weeks and 6 control mice received drinking water only. Tumor size was monitored using dial calipers. At the end of the experiment, blood samples, tumors, hearts, and limb muscles were collected and measured for VEGF expression using ELISA and capillary density (CD) using CD31 immunohistochemistry. Compared to the control, EGCG treatment significantly reduced tumor weight (2.9±0.5 vs. 5.9±1.1 g; P<0.01; n=6), melanoma CD (117±9 vs. 167±23; P<0.01), and melanoma VEGF expression (32±1.5 vs. 42±2 pg/mg; P < 0.01), respectively. Also EGCG had no effects on body weight, heart weight, angiogenesis or VEGF expression in the heart and skeletal muscle of mice. EGCG (20-50 μg/ml) significantly inhibited the proliferation, migration, VEGF expression, and the activation of HIF-1α and NFκB in cultured B16F10 cells, respectively. These findings support the hypothesis that EGCG, a major green tea polyphenol, directly targets tumor cells and tumor vasculature, thereby inhibiting tumor growth, proliferation, migration, and angiogenesis of melanoma, and that the down-regulation of VEGF expression by EGCG is associated with the inhibition of HIF-1α and NFκB activation. EGCG has great potential as a chemopreventive agent because it has no effect on angiogenesis in normal tissue and has low toxicity.

**Keywords:** Melanoma, angiogenesis, proliferation, migration, EGCG, green tea polyphenols, VEGF, HIF-1α, NFκB, and capillary density in the heart.

## INTRODUCTION

Skin cancer affects an estimated 3.5 million Americans each year [1] and the incidence of melanoma has been increasing steadily over the past few decades in most Western countries [2]. Melanoma is less common than other skin cancers, but it causes the majority (75%) of skin cancer-related death [3]. Although surgical excision can eliminate melanomas if the lesions are removed at the earliest stages of the disease, a significant number are not detected or excised early, leading to rising incidence and mortality rates [4]. Also, individuals with a history of melanoma have 12 times the likelihood of developing a second primary lesion compared with patients without a history of melanoma [5]. Moreover, there is no effective therapy for high risk melanoma patients to prevent relapse or additional tumors [6]. Therefore, chemo-prevention strategies are needed. Chemopreventive agents act at multiple steps of various pathways to block carcinogenesis such as tumor incidence, onset and progression. Chemoprevention is primarily attributed to the use of naturally occurring agents, which are found in the food we consume and hence not toxic and easily available.

Next to water, tea is the most commonly consumed beverage worldwide because of its characteristic aroma, flavor and health benefits [7]. Green tea contains more than 40% polyphenols, the large majority of which are flavonoid monomers called catechins (30% in green tea) and flavonols (2% in green tea) [8]. The natural product epigallocatechin-3-gallate (EGCG) accounts for 50-80% of catechins in green tea, representing 200–300 mg in a brewed cup of green tea [9]. EGCG is the major green tea polyphenol contributing to its beneficial therapeutic actions including anti-oxidant, anti-inflammatory, anti-cancer, and immunomodulatory effects [10-12]. Studies conducted on cell-culture systems and animal models as well as human epidemiological studies show that EGCG in green tea could afford protection against a variety of cancer types [13]. Limited data are currently available from EGCG chemoprevention trial. EGCG delivered in the form of capsule (200 mg p.o.) for 12 weeks has been reported to be effective in the patients with human papilloma virus-infected cervical lesions

*Address correspondence to this author at the Cancer Institute, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216-4505, USA; Tel: 601-815-3402; Fax: 601-984-2981; E-mail: jgu@umc.edu

ISSN: 1929-2260 / E-ISSN: 1929-2279/14 

[14]. Oral EGCG at a dose of 2000 mg (200 mg per capsule supplied by the NCI) twice daily for up to 6 months was well tolerated by patients with chronic lymphocytic leukemia in this phase 2 trial, and durable declines in the absolute lymphocyte count and/or lymphadenopathy were observed in the majority of patients [15]. Many studies have shown that the oral administration of green tea polyphenols (GTPs) in drinking water inhibits UV radiation-induced non-melanoma skin cancer in mice in terms of tumor incidence, tumor multiplicity and tumor growth [16, 17]. These molecular targets of GTPs include DNA repair [16, 17], stimulation of the immune system [19, 20], anti-inflammatory effects [21], and anti-oxidant activity [22]. Although EGCG inhibits melanoma cell growth [23] and invasion/migration [24], the beneficial effects of GTPs on melanoma are not well studied and less understood.

The growth and expansion of a tumor is mainly dependent on angiogenesis, the formation of new capillaries from pre-existing blood vessels. Avascular tumors can't grow beyond a maximum size of 1 to 2 $mm^3$ without neovascularization and may be eliminated by the immune system [25]. Angiogenesis requires stimulation of vascular endothelial cells through the release of angiogenic factors. Of these, the vascular endothelial growth factor (VEGF) is the most critical regulator in the development of the vascular system and is commonly overexpressed in a variety of human solid tumors including melanoma [26]. Cancer cells are under greater hypoxia and oxidative stress than normal cells.

Oxygen radicals and hypoxia co-operatively promote tumor angiogenesis [25], by causing the activation of HIF-1 which stimulates VEGF expression. In addition, oxygen radicals activate NFκB that also increases VEGF expression. VEGF is a key angiogenic factor that stimulates the growth of tumors including melanoma. We previously reported that VEGF-mediated angiogenesis promotes melanoma tumor growth in mice [28]. We believe that EGCG can block highly activated NFκB, HIF-1α, and VEGF expression in melanoma. Therefore, we hypothesize that EGCG directly targets both tumor cells and tumor vasculature, inhibiting tumor growth, proliferation, migration, and angiogenesis of melanoma, through the inhibition of HIF-1α and NFκB activation as well as VEGF expression. Also, EGCG treatment has no significant effects on the body weight, heart weight, or angiogenesis/VEGF expression in normal tissues such as the heart and skeletal muscle.

To test this hypothesis, the present study aimed to determine the following: 1) whether oral EGCG inhibits melanoma tumor growth, tumor angiogenesis, and VEGF expression in an immunocompetent mouse melanoma (B16F10) model; 2) whether oral EGCG treatment affects angiogenesis and VEGF expression in normal tissues such as the heart and skeletal muscle in the same mice; and 3) whether EGCG inhibits proliferation, migration, VEGF expression, and the activation of HIF-1α and NFκB in cultured mouse melanoma (B16F10) cells.

## MATERIALS AND METHODS

### Chemicals and Cell Line

EGCG was purchased from Sigma Chemical Co. (St. Louis, MO). The mouse melanoma (B16F10) cells were ordered from American Type Culture Collection (ATCC) (Manassas, VA 20110 USA). The B16F10 cells were maintained as monolayer cultures in M199 media (GIBCO) supplemented with 10% FBS (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, and incubated at 37˚C in a humidified 5%$CO_2$/air injected atmosphere.

### Animal Protocols

The protocols were carried out according to the guidelines for the care and use of laboratory animals implemented by the National Institutes of Health and the Guidelines of the Animal Welfare Act and were approved by the University of Mississippi Medical Center's Institutional Animal Care and Use Committee. 12 male C57BL/6 mice at 6-7 weeks of age were purchased from Jackson Laboratory (Bar Harbor, Maine). The mice were allowed to acclimate for 1 week with standard chow diet (Teklad, Harlan Sprague Dawley; Indianapolis, IN) and tap water before beginning the experiments. The eight week old male mice (n = 12) were inoculated with 5 x 10^5 B16F10 cells suspended in 100 μl of phosphate-buffered saline into the right proximal dorsal of the back in mice. Then, 6 mice received EGCG (25 mg/50 ml) in drinking water for 4 weeks and 6 control mice received drinking water only. Each mouse (20 g) usually drank 2 to 4 ml of water per day. Therefore, EGCG was given around 50 to 100 mg/kg/day to the experimental mice. The body weight of the mice was monitored weekly. Tumor size was monitored every other day in two perpendicular dimensions parallel with the surface of the mice using dial calipers. At the end of the experiment, blood samples, tumors, hearts and limb muscles were

collected to measure VEGF expression using ELISA and average microvascular density (AMVD) or capillary density (CD) using CD31 immunohistochemistry.

### Morphometric Analysis of Angiogenesis in Tumors, Hearts, and Limb Muscles

The quantification of blood vessels in the mouse melanoma tumors, hearts and limb muscles was determined with the modification of a previously reported method [29, 30]. Briefly, the tissues were fixed in 4% neutrally buffered paraformaldehyde. For the left ventricle of the heart and limb muscle samples, consecutive thin transverse cryosections (5 μm) were cut along the base-apex axis. Consecutive thin cryosections (5 μm) of OCT compound (Sakura Finetek, Torrance, CA) embedded tissue samples were fixed in acetone at 4°C for 10 min. After washing in phosphate buffered saline (PBS), the sections were first treated with 3% $H_2O_2$ for 10 minutes to block endogenous peroxidase activity and then were blocked with normal rabbit serum. Next, the sections were washed in PBS and incubated with rat anti-mouse CD31 (PECAM-1) monoclonal antibody (BD Pharmingen, San Diego, CA) at a 1:200 dilution overnight at 4°C. Negative controls were incubated with the rat serum IgG at the same dilution. All sections were washed in PBS containing 0.05% Tween-20, and were then incubated with a 2nd antibody, mouse anti-rat IgG (Vector laboratories, Burlingame, CA) at a 1:200 dilution for 1 hour at room temperature, again followed by washing with PBS containing 0.05% Tween-20. The sections were incubated in a 1:400 dilution of Extravadin Peroxidase (Sigma, St. Louis, MO) for 30 min. After washing in PBS containing 0.05% Tween-20, the sections were incubated in peroxidase substrate (Vector laboratories, Burlingame, CA) for 5 min. The sections were washed in PBS containing 0.05% Tween-20 and were counterstained with hematoxylin. A positive reaction was indicated by a brown staining. The microvascular vessels or capillary density (CD) were quantified by manual counting under light microscopy. A microscopic field (0.7884 $mm^2$) was defined by a grid laced in the eye-piece. At least 20 microscopic fields were randomly acquired from each tumor for analysis. Any endothelial cell or cell cluster showing antibody staining and clearly separated from an adjacent cluster was considered to be a single, countable microvessel or capillary. The value of the average microvascular density (AMVD) or capillary density (CD) was determined by calculating the mean of the vascular counts per $mm^2$ obtained in the microscopic fields for each tissue sample.

### Measurements of Protein Levels of VEGF by ELISA

Protein levels of VEGF in plasma, melanoma tumor, the heart, the limb muscle, and the medium cultured with B16F10 cells were determined using mouse VEGF ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The total proteins of melanoma tumor, the heart, the limb muscle, and cultured B16F10 cells were extracted using NE-PER Cytoplasmic Extraction Reagents (Pierce, Rockford, IL) according to the manufacturer's protocol. The total protein concentration of these tissue extractions was determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). The protein concentrations of VEGF were normalized and expressed as pictograms per milligram of total tissue or cell extraction protein.

### Proliferation Assay of Cultured Melanoma Cells

The B16F10 cells were seeded into 6-well tissue culture plates using M199 Medium (GIBCO) supplemented with 10% FBS (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, and incubated at 37°C in a humidified 5%$CO_2$/air injected atmosphere. When the monolayer reached about 80% confluence, the cells were washed with PBS and incubated with fresh M199 Medium with 10% FBS in the absence or presence of EGCG (0, 20, 40, or 80 μg/ml) for 18 hours. 3H-thymidine incorporation assay was used to determine the cell proliferation during the last 6 hours of incubation as previously described [31].

### Migration Assay

Migration was determined using BD BioCoat Matrigel Invasion Chamber (BD Bioscience Discovery Labware, Sedford, MA) according to a previous study, in which only invasive cells digested the matrix and moved through the insert membrane [32]. 1 × 10^5 B16F10 cells per well in 0.5 ml M199 medium were seeded in the matrigel-coated upper compartment (insert) of a Transwell (24-well format, 8-μm pore) in the absence or presence of EGCG (50 μg/ml). Medium with 10% FBS was added to the lower part of the well. After overnight incubation at 37°C and 5% CO2, cells on the upper surface of the insert were removed using a cotton wool swab. Migrated cells on the lower surface of the insert were stained using DiffQuit (Dada Behring, Düdinen, Switzerland). Images of migrated cells were taken and the number of migrated cells was counted using a microscope (Leica, Germany) in a 20× objective.

### HIF-1α and NFκB Activation (Motif Binding) Assays

We determined HIF-1α and NFκB activation in cultured B16F10 cells in the presence or absence of EGCG (50 μg/ml) to investigate whether the down-regulation of VEGF by EGCG is associated with the inhibition of HIF-1α and NFκB activation (n = 6). The nuclear proteins were extracted by using an Active Motif (Carlsbad, CA) nuclear extract kit. 20 μg of nuclear proteins from each sample were used in the TransAM HIF-1α or NFκB p65 kit (Active Motif), which measures the binding of activated HIF-1α or NFκB to its consensus sequence attached to a microwell plate, according the manufacturer's instructions.

### Statistical Analysis

All determinations were performed in duplicated sets. Where indicated, data is presented as mean ± SE. Statistically significant differences in mean values between the two groups were tested by an unpaired Student's t-test. Linear regression was performed by the correlation analysis between two continuous variables. A value of $P < 0.05$ was considered statistically significant. All statistical calculations were performed using SPSS software (SPSS Inc., Chicago, IL).

## RESULTS

### EGCG Significantly Inhibits the Progression of Melanoma Growth in an Immune-Competent Mouse Model

We used a mouse melanoma model that mimics the human disease, and the mouse melanoma (B16F10) cells were injected into the right proximal dorsal of the back in male immune-competent mice (C57BL/6). Immediately after the inoculation of B16F10 cells, the eight week old male mice (n = 6) were given EGCG at 50 to 100 mg/kg/day in drinking water for four weeks and the control group (n = 6) was given regular drinking water only. As indicated in Figure **1A**, the tumor volume was significantly reduced in the EGCG-treated group compared to the control group two weeks after the melanoma inoculation. At the end of experiment, tumor volume was reduced by 67% ($P<0.01$) in EGCG-treated group compared to the control group (Figure **1A**), and was consistent with the reduction in tumor weight (Figure **1B**) in EGCG-treated group compared to the control group (2.9±0.5 vs. 5.9±1.1 g; n=6; $P<0.01$). Clearly, EGCG treatment at 50 to 100 mg/kg/d in drinking water significantly inhibited the progression of melanoma growth in the male immune-competent mice by decreasing the tumor size and reducing the growth curve of melanoma. However, there was no significant difference in body weight, heart weight, and kidney weight between the EGCG-treated mice and the control mice.

### EGCG Treatment Suppresses Melanoma Angiogenesis and Melanoma VEGF Expression, and Circulating VEGF in Mice

Representative images of CD31 staining of the melanomas showed that the EGCG-treated tumor had significantly lesser capillary density than the control tumor (Figure **2A**). The brown staining (CD31) indicates the microvascular vessels or capillaries. Morphometric analysis (Figure **2B**) demonstrated that EGCG significantly decreased melanoma capillary density (number of capillaries per $mm^2$ area), compared to the control group (117±9 vs. 167±23; $P<0.01$; N = 6). These results also suggest that a pronounced decrease in tumor angiogenesis is

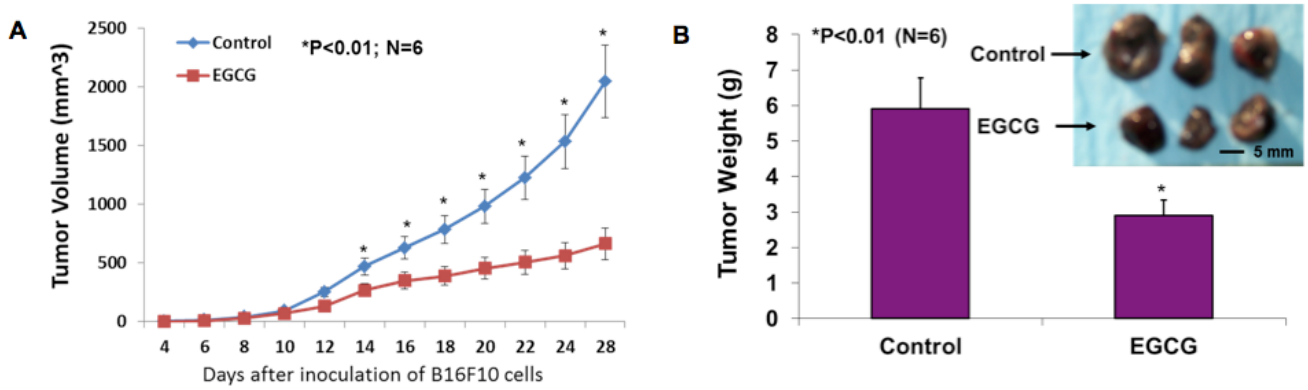


**Figure 1:** The treatment of EGCG significantly suppressed the mouse melanoma growth curve of tumor volume (**A**) and tumor weight (**B**) over the control in male immune-competent mice. After 4 weeks of oral EGCG (50-100 mg/kg/d) in drinking water, the tumor volume was reduced by 67% ($P<0.01$) in EGCG-treated group compared to the control group, and was consistent with the reduction in tumor weight in EGCG-treated group compared to the control group (2.9±0.5 vs. 5.9±1.1 g; $P<0.01$; n=6).

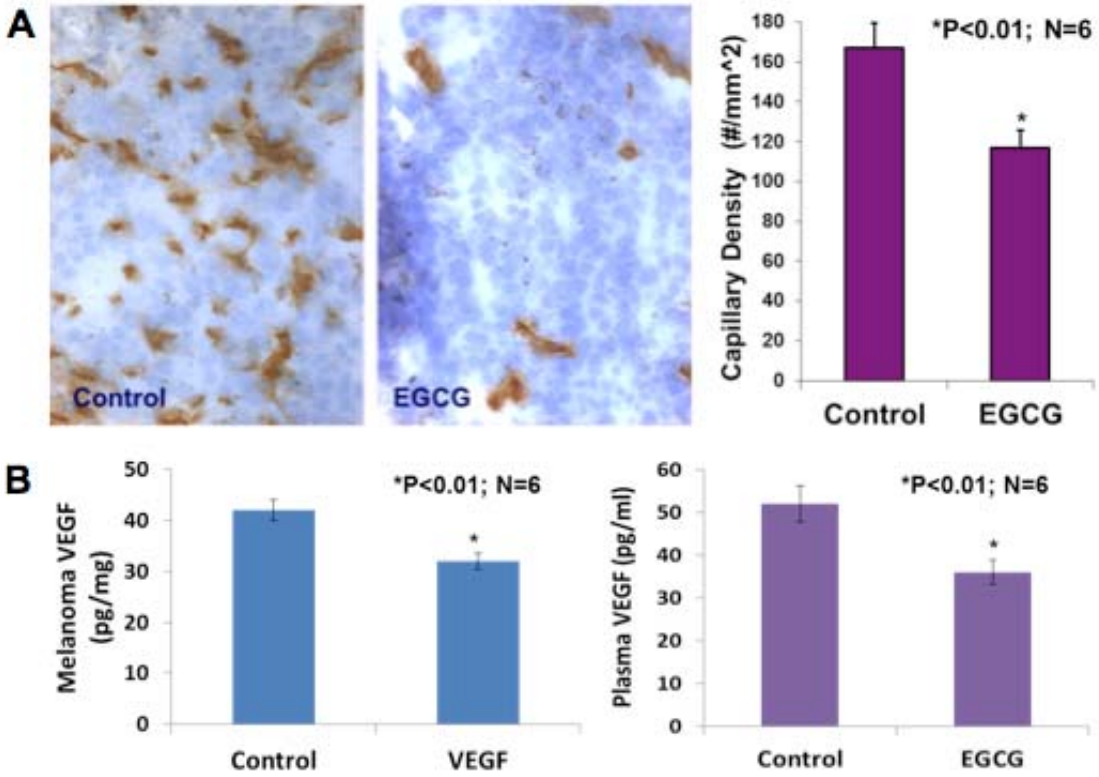


**Figure 2:** Oral EGCG significantly reduced melanoma capillary density (Panel **A**: 117±9 vs. 167±23; P<0.01; n=6), plasma VEGF levels (Panel **B**: 36 ± 3 vs. 52 ± 4 pg/ml; P < 0.01; n=6), and melanoma VEGF expression (Panel **B**: 32 ± 1.5 vs. 42 ± 2 pg/mg; P<0.01; n=6) over the control mice, respectively. The digital images show CD31 immunohistochemistry staining in OCT-embedded cryosections of mouse melanomas obtained from a control or EGCG-treated (**A**) mouse.

associated with a decrease in melanoma tumor size in the mice treated with EGCG compared to those in the control mice. Figure **2B** also demonstrated that EGCG treatment reduced plasma VEGF levels over the control mice (36 ± 3 vs. 52 ± 4 pg/ml; n = 6; P < 0.01) and tumor VEGF expression over the control mice (32 ± 1.5 vs. 42 ± 2 pg/mg; n = 6; P < 0.01). These findings suggest that the inhibition of tumor angiogenesis in mice by EGCG is due to the down-regulation of VEGF.

### EGCG Treatment Directly Inhibits Proliferation and Migration of Melanoma Cells

We used a 3H-thymidine incorporation assay to determine the effects of EGCG on the proliferation of cultured mouse melanoma cells (B16F10). Figure **3** showed that B16F10 cells treated with EGCG caused a dose-related decrease in 3H-thymidine incorporation, decreasing by 21% at 20 µg/ml and by 75% at 40 µg/ml, compared to the control group (P < 0.01; n = 6). However, 80 µg/ml of EGCG did not cause any further significant decrease in the proliferation. This flat phase may suggest that some of the many different signals controlling the proliferation of melanoma cells are not inhibited by EGCG. We examined the inhibitory effect of EGCG on B16F10 cell migration using BD BioCoat Matrigel Invasion Chamber. Figure **4** demonstrates that EGCG at 50 µg/ml significantly reduced migration of melanoma cells by 57%, compared to the control group (31±3 vs. 73±7 cell number per view (0.79 mm^2; P < 0.01; n = 6). These *in vitro* findings illustrate that EGCG can directly target melanoma cells by inhibiting its proliferation and migration.

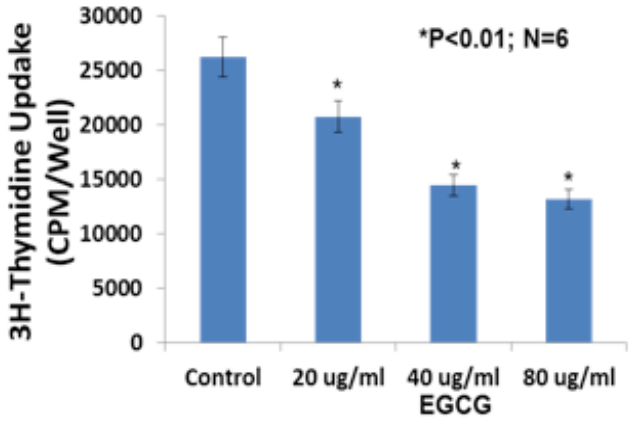


**Figure 3:** EGCG caused a dose-related inhibition of 3H-thymidine incorporation in cultured melanoma cells (B16F10), decreasing by 21% at 20 µg/ml and by 75% at 40 µg/ml, compared to the control group (P<0.01; n=6). EGCG (80 µg/ml) did not cause any further significant decrease in the proliferation.

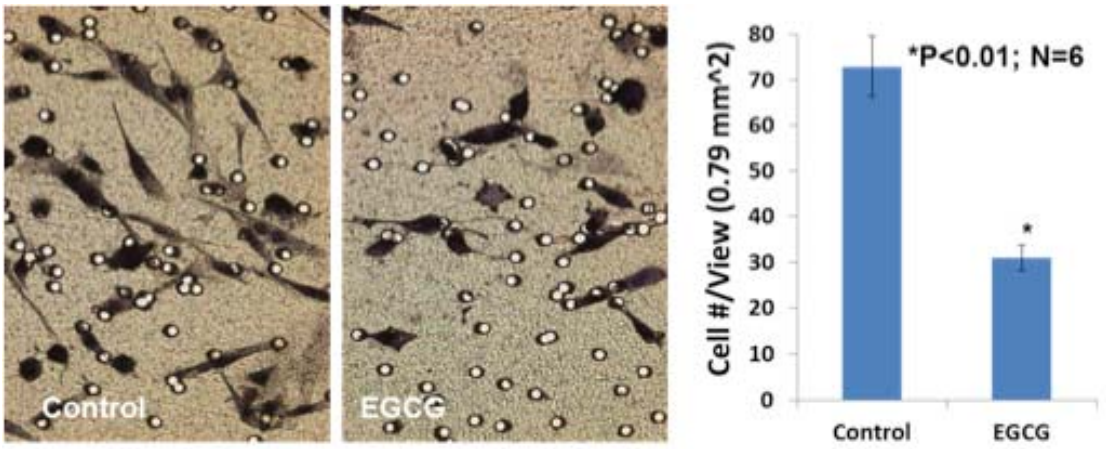


**Figure 4:** EGCG (50 μg/ml) significantly inhibited the melanoma cell invasion or migration in BD BioCoat Matrigel Invasion Chamber, compared to the control group (31±3 vs. 73±7 cell number per view (0.79 mm^2; P<0.01; n=6). The images show the migrated melanoma cells.

### The Down-Regulation of VEGF Expression by EGCG is Associated with the Inhibition of HIF-1α and NFκB Activation

HIF-1 and NFκB pathways are highly activated in melanoma [33], and they co-operatively promote tumor angiogenesis by increasing VEGF expression. We used a VEGF ELISA kit and HIF-1α and NFκB activation (Motif Binding) assays to determine whether EGCG could suppress HIF-1α and NFκB activation and VEGF expression in cultured mouse melanoma (B16F10) cells. Figure **5** demonstrates that EGCG (50 μg/ml) significantly inhibits VEGF expression (2365 ± 164 vs. 3214 ± 225 pg/mg; P < 0.01; n = 6) in cultured B16F10 cells, compared to the control. In the same experiment, EGCG (50 μg/ml) also significantly suppressed the activation of HIF-1α (0.25 ± 0.02 vs. 0.48 ± 0.05 OD450; P < 0.01; n = 6) and NFκB (2.41 ± 0.15 vs. 3.35 ± 0.21 OD450; P < 0.01; n = 6), compared to the control, respectively. These results suggest that the inhibition of HIF-1α and NFκB activation contributes to the down-regulation of VEGF expression.

### EGCG Treatment has No Effects on Angiogenesis and VEGF Expression in Normal Tissues such as the Heart and Limb Muscles in Mice

The data showed that there was no significant difference in body weight (23.41 ± 0.52 vs. 23.94 ± 0.58; P = 0.9346; n = 6), heart weight (84.9 ± 11.3 vs. 85.4 ± 10.4 mg; P = 0.3546; n = 6), or kidney weight (236.6 ± 9.1 vs. 239.2 ± 8.8 mg; P = 0.3824; n = 6) between the EGCG-treated mice and the control mice. Figure **6A** showed that EGCG treatment did not affect the capillary density (3284 ± 331 vs. 3225 ± 321 #/mm$^2$; P = 0.6324; n = 6) analyzed by CD31 immunochemistry and morphometric analysis, or VEGF

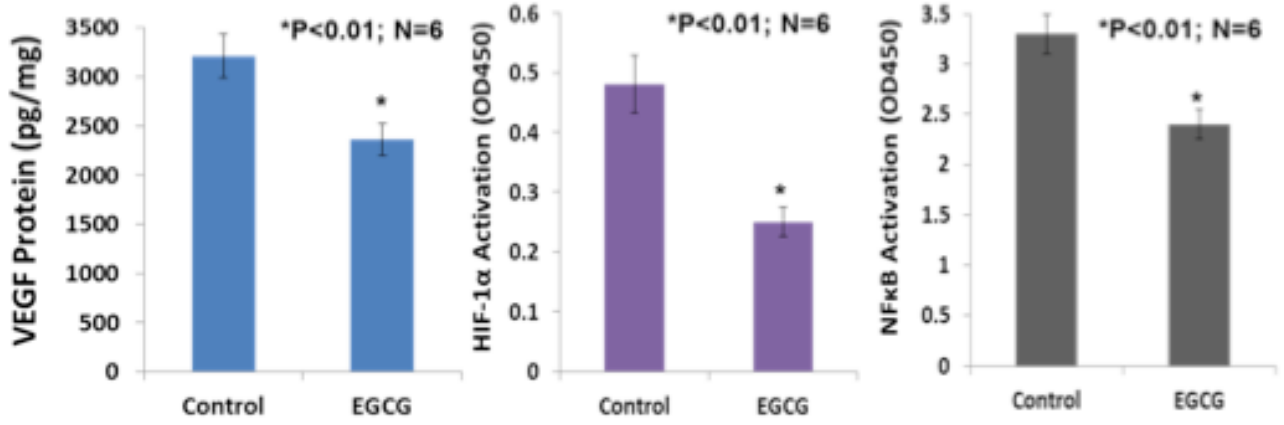


**Figure 5:** EGCG (50 μg/ml) significantly inhibited VEGF expression (2365±164 vs. 3214±225 pg/mg; P<0.01; n=6), the activation of HIF-1α (0.25±0.02 vs. 0.48±0.05 OD450; P<0.01; n = 6) and NFκB (2.41±0.15 vs. 3.35±0.21 OD450; P<0.01; n=6) in cultured mouse melanoma (B16F10) cells, compared to the control, respectively.

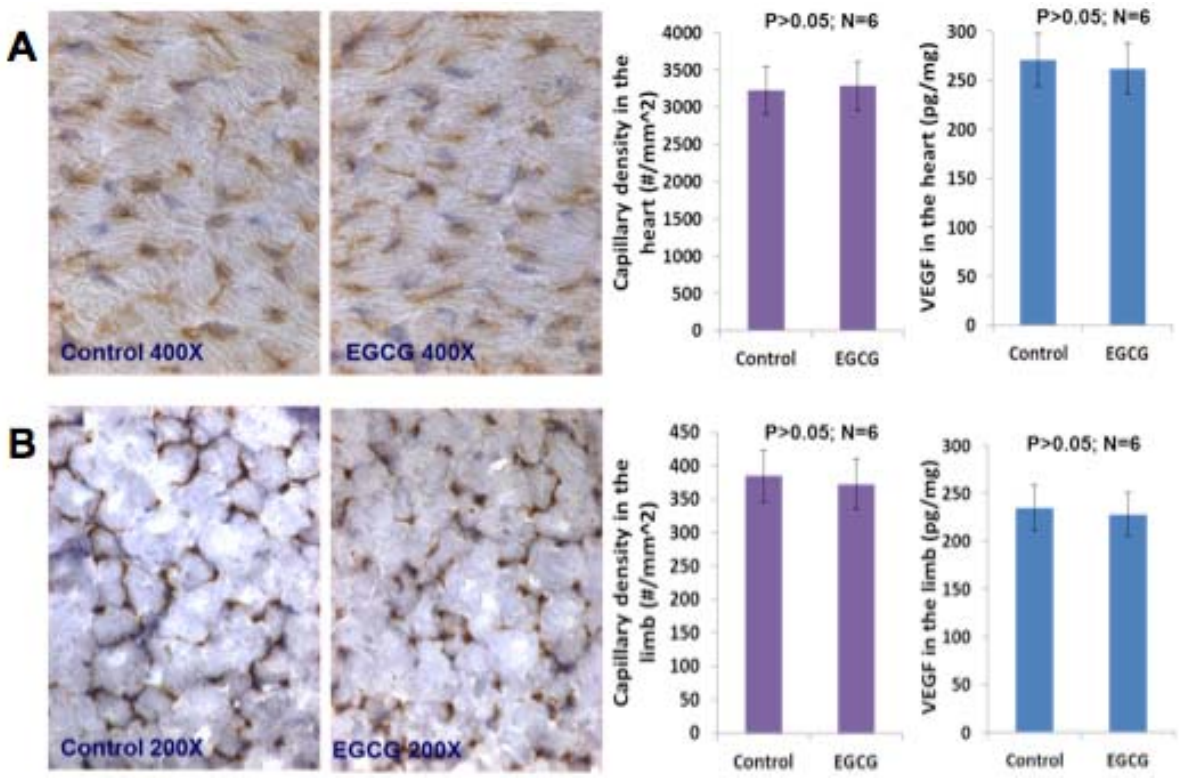


**Figure 6:** EGCG treatment did not affect the capillary density (number of capillaries/ mm^2 area) (3284±331 vs. 3225±321 #/mm^2; P=0.6324; n=6) or VEGF expression (262±22 vs. 271±25 pg/mg; P=0.5717; n = 6) in the mouse heart, compared to the control group (Panel **A**). There was no significant difference in the capillary density (385±41 vs. 373±39 #/mm^2; P=0.5912; n=6) or VEGF expression (235±21 vs. 228±19 pg/mg; P=0.6752; n=6) in the limb skeletal muscles between the EGCG-treated mice and the control mice (Panel **B**). The digital images show CD31 immunohistochemistry staining in OCT-embedded cryosections of the heart (Panel **A**) and the limb muscle (Panel **B**) of control mouse and EGCG-treated mouse, respectively.

expression (262 ± 22 vs. 271±25 pg/mg; P=0.5717; n = 6) determined by ELISA in the mouse heart, compared to the control group, respectively. Figure **6B** showed that there was no significant difference in the capillary density (385 ± 41 vs. 373 ± 39 #/mm$^2$; P = 0.5912; n = 6), or VEGF expression (235 ± 21 vs. 228 ± 19 pg/mg; P = 0.6752; n = 6) in the limb skeletal muscles between the EGCG-treated mice and the control mice, respectively. These findings illustrate that EGCG does not significantly affect angiogenesis and VEGF expression in the normal tissues such as the heart and limb skeletal muscles.

## DISCUSSION

Melanoma is a highly malignant cancer with a potent capacity to metastasize distantly contributing to its higher mortality. More effective, minimally toxic systemic agents are needed to decrease the morbidity and mortality of melanoma. However, the investigations of green tea polyphenols (GTPs) or EGCG in melanoma using animal models are very limited, and the role of GTPs or EGCG in melanoma treatment and prevention is poorly understood.

The major new findings from the present investigation include: 1) oral EGCG significantly inhibits the progression of mouse melanoma tumor growth in male immune-competent mice; 2) EGCG significantly suppresses melanoma tumor angiogenesis and tumor VEGF expression in these mice; 3) EGCG treatment does not significantly affect angiogenesis and VEGF expression in the normal tissues such as the heart and skeletal muscles; 4) EGCG directly inhibits proliferation and migration of cultured mouse melanoma cells; and 5) the down-regulation of VEGF expression by EGCG is associated with the inhibition of HIF-1α and NFκB activation. These findings support the hypothesis that EGCG, a major green tea polyphenol directly targets both melanoma tumor cells and tumor vasculature, thereby inhibiting tumor growth, proliferation, migration, and angiogenesis of melanoma associated with inhibiting HIF-1α and NFκB activation as well as VEGF expression. Also, EGCG treatment has no adverse effects on angiogenesis and VEGF expression in normal tissues such as the heart and skeletal muscle.

An important finding of this study is that oral EGCG treatment at 50 to 100 mg/kg/day in drinking water

significantly slows a growth curve of melanoma in C57BL/6 male mice compared to the control group, which is characterized by 67% and 51% reduction in the tumor volume and tumor weight, respectively. Clearly, oral EGCG treatment is very effective in suppressing progression of melanoma in a wild type immune-competent mouse model. Ullmann *et al*. reported that peak plasma concentrations were greater than 3μg/ml after oral dose of 1600 mg EGCG in healthy human subjects [34]. This suggests that drinking more than 10 cups of green tea may be necessary to maintain a plasma concentration of EGCG equivalent to that used to produce a dose of 50 μM *in vitro*. We believe that an oral dose of 50 to 100 mg/kg/day in humans can reach the effective plasma concentrations of EGCG against melanoma. Kaegi [35] suggested a daily intake of 13 cups of green tea as a chemopreventive measure. The currently market-available extracts of green tea polyphenols contain 98% polyphenols and 40% EGCG. Recent methods have been developed for the stereo-selective total synthesis of EGCG and structurally related catechins [36]. These products could provide new sources of chemoprevention agents for biomedical use. However, the further evaluations and clinical trials for these products are necessary.

Cancer cells are under greater hypoxia and oxidative stress than normal cells. In a hypoxic microenvironment melanoma cells show increased HIF-1α expression and induction of angiogenesis to acquire an adequate blood supply [37]. Interestingly, the transcription factor NFκB is constitutively up-regulated in malignant melanoma [38]. Tumor cells overproduce reactive oxygen species (ROS) by alterations of their metabolic pathways in tumor cells [39], an inadequate tumor vascular network [40], and macrophage infiltration of the tumor [41].

Melanomas support their growth by stimulating angiogenesis, but blood flow within these new vessels is often chaotic, causing periods of hypoxia followed by reperfusion. The generation of ROS by reperfusion further causes oxidative stress within melanomas. Melanoma rapidly outgrows its blood supply, leading to hypoxia and glucose deprivation and hypoxia which induces oxidative stress within cancer cells [42]. Clearly, hypoxia and oxidative stress are found together within the melanoma, which synergistically augment VEGF production. Oxygen radicals and hypoxia co-operatively promote tumor angiogenesis [40] by causing the activation of HIF-1 and NFκB which stimulates VEGF expression. Thus, any compound which can block HIF-1 and NFκB pathways can significantly inhibit VEGF expression and angiogenesis in carcinomas including melanomas.

The present study shows that the significant inhibitions of tumor growth and tumor angiogenesis of melanomas in male immune-competent mice by EGCG are associated with suppression of the activation of HIF-1α and NFκB, which decreases VEGF expression in melanoma cells. VEGF is a key angiogenic factor and the presence of VEGF receptors on melanoma cells may suggest an autocrine effect of VEGF that stimulates the growth of tumors including melanoma [28, 43-46]. VEGF overexpression [28, 43, 44] and the activation of HIF-1α and NFκB pathways in melanoma are strongly linked to the rapid growth of tumors and a worse prognosis [37, 38, 43-46]. Reactive oxygen species and hypoxia co-operatively promote tumor angiogenesis, activate HIF-1α and NFκB pathways, and over-express VEGF in melanoma [37, 38]. The present findings indicate that EGCG significantly inhibits VEGF expression associated with suppressing the activation of HIF-1α and NFκB pathways, thereby inhibiting tumor growth, proliferation, migration, and angiogenesis of melanoma. Our results are supported by the previous findings as follows: 1) EGCG inhibited melanoma cell growth [23] and invasion/migration [24]; 2) EGCG suppressed tumor growth by blocking the induction of VEGF in human colon carcinoma cells [47]; 3) EGCG inhibited VEGF/VEGFR axis by suppressing the expression of HIF-1α in human colorectal cancer cells [48]; and 4) EGCG inhibited cancer progression by decreasing NFκB activation [49].

The progression stage is the final phase of cancer development in which an uncontrolled growth of cancer cells occurs. In this stage cancer cells are under greater hypoxia and oxidative stress, and many transcription factors, such as HIF-1α and NFκB, are activated. This leads to transmission of aberrant signals resulting in abnormal functions such as tumor angiogenesis, cancer invasiveness and metastasis. Present findings illustrate that EGCG can inhibit multiple key cellular signals which inhibits tumor angiogenesis and melanoma progression. Also, accumulating evidence shows that EGCG can target all stages of cancer development by blocking multiple cellular proteins involved in diverse cellular signal transduction pathways: proliferation, differentiation, apoptosis, angiogenesis and metastasis [50]. In future studies, we will investigate the therapeutic potentials of EGCG combined with a VEGF receptor inhibitor, Notch inhibitor, HIF-1 inhibitor, and/or NFκB blocker in melanoma therapy.

Interestingly, the present study shows that oral EGCG treatment significantly inhibits angiogenesis, VEGF expression, and growth in melanoma, but has no such effects on normal tissues such as the heart and limb muscles in the same mice. The different effects of EGCG in tumor and normal tissues can be explained by the fact that cancer cells are under greater hypoxia and oxidative stress than normal cells. VEGF expression and angiogenesis are very stable in normal matured tissues because they are regulated by metabolic balance within the tissue. However, angiogenesis is stimulated by significantly increased VEGF levels, and activated HIF-1α and NFκB pathways in cancer. The finding of EGCG selectively inhibiting melanoma angiogenesis but not physiologic angiogenesis is supported by the report that EGCG inhibited migration and phosphorylation of Akt in melanoma-associated endothelial cells but not in normal skin endothelial cells [51]. We also found that there was no significant difference in the body weight, heart weight, or kidney weight between EGCG-treated mice and the control mice. This is an exciting possibility, because EGCG is a drug of low toxicity. EGCG and green tea polyphenols (GTPs) could be excellent chemoprevention agents.

In conclusion, our results indicate that oral administration of EGCG, a major green tea polyphenol, significantly inhibits tumor growth and angiogenesis in melanoma, but has no effect on angiogenesis in the heart and limb muscles in an immune-competent mouse model using mouse melanoma (B16F10) cells. EGCG directly suppresses the proliferation and migration of cultured mouse melanoma cells. These anticancer effects of EGCG seem to be mediated by blocking multiple intracellular signaling cascades associated with inhibiting the HIF-1α and NFκB pathways. The mechanistic advance of EGCG on inhibiting tumor angiogenesis is very unique, as EGCG does not target angiogenesis in normal tissue. Accumulating evidence indicates that EGCG displays a vast array of cellular effects involved in all stages of cancer development. The multiple targets on cancer and minimal side effects of EGCG will lead to a successful targeted therapy and/or chemoprevention for cancers including melanoma. The potential therapeutic targets of EGCG in cancer therapy need to be further explored. Our next step is a clinical trial for EGCG and/or green tea polyphenols in melanoma therapy and/or chemoprevention. The combination of EGCG with other targeted compounds such as a VEGF receptor inhibitor, Notch inhibitor or HIF-1 inhibitor could lead to a very effective specific targeted melanoma therapy.

## ACKNOWLEDGEMENT

This work was supported by the National Institute on Alcohol Abuse and Alcoholism Grant AA-013821 and the National Heart, Lung, and Blood Institute Grant HL-51971, and a research fund from University of Mississippi Medical Center Cancer Institute.

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Received on 17-10-2013 Accepted on 22-11-2013 Published on 13-02-2014

DOI: http://dx.doi.org/10.6000/1929-2279.2014.03.01.3