

A Bivalent Role of Genistein in Sprouting Angiogenesis

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ABSTRACT

The effects of genistein on angiogenesis remain poorly understood. Some studies claim an antiangiogenic effect and others claim a pro-angiogenic one. Thus, the aim of this study was to determine if genistein may exhibit bivalent angiogenic effects. To address this question, genistein angiogenic modulatory effects were examined using an *in vitro* 3D angiogenesis model using human umbilical vein endothelial cells. In this model, a bivalent effect of genistein was demonstrated on sprouting angiogenesis, with angiogenic stimulation at low concentrations (0.001–1 μ M) and inhibition at higher ones (25–100 μ M). Enhancement of the endothelial tube formation correlated with an increase in human umbilical vein endothelial cell metabolic activity and proliferation. Inhibition of angiogenesis correlated with a decreased metabolic activity, proliferation, and migration. Moreover, high concentrations of genistein influenced human umbilical vein endothelial cell morphology. Expression of genes involved in the angiogenic process in response to genistein was measured to study the mechanism of action. Secretome profiling revealed that angiogenic regulators were modulated with genistein treatment. These results suggested a bivalent effect of genistein on human umbilical vein endothelial cell growth and angiogenesis, and further investigations on the benefit of genistein for cancer chemoprevention, cancer treatment, or pro-angiogenic therapies have to be carefully considered.

Introduction

Angiogenesis refers to the formation of new blood vessels from preexisting ones. It is crucial for survival and normal physiology, and plays important roles in wound healing. However, if deregulated, it may lead to tumor growth and metastasis [1]. Therefore, targeting tumor angiogenesis has been used as a strategy to reduce the tumor burden and prolong the overall survival of cancer patients [2]. Antiangiogenic compounds include monoclonal antibodies such as bevacizumab, which target the VEGF, and tyrosine kinase inhibitors such as sorafenib, acting on the VEGF receptor. These agents are currently used in the clinic for the treatment of a wide range of tumors such as breast, gastric, pancreatic, hepatocellular, lung, and neuroendocrine tumors. However, these therapies demonstrated not to be curative as several mechanisms of resistance arose following treatment [2].

During the past few decades, phytochemicals have raised a substantial interest for their therapeutic potential in cancer che-

moprevention, and among those is the phytoestrogen genistein predominantly found in soy [3,4]. Genistein demonstrated antitumor effects in prostate, melanoma, and breast mouse models, an effect that was later linked to antiangiogenic properties [5–9]. In contrast, a recent study demonstrated an effect of genistein on regenerative angiogenesis in a mouse model [10]. Furthermore, several studies showed that genistein exhibited estrogenic effects, whereas multiple other studies indicated that genistein had antiestrogenic ones [11]. This may play a role in the genistein-mediated effect on the prevention or promotion of breast cancer. This bivalent behavior is of medical significance as it is a common feature in numerous pathological conditions, ranging from neoplasia to cardiovascular protection and repair. Therefore, it is of prime importance to understand the effects of genistein on ECs to assess its safety for future therapeutic purposes. The pur-

* Mark E. Issa and Gilles Carpentier contributed equally to this work.

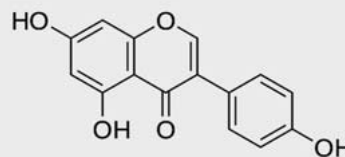
ABBREVIATIONS

ANGPT-2	angiopoietin 2
bFGF	basal fibroblast growth factor
CAM	calcein AM
CD31	cluster of differentiation 31
EBM-2	endothelial basal medium 2
ECs	endothelial cells
ECGM-2	endothelial cell growth medium 2
EGF	epidermal growth factor
EndMT	endothelial-to-mesenchymal transition
EPCs	endothelial progenitor cells
FGM	fibroblast growth medium
FITC	fluorescein isothiocyanate
HUVECs	human umbilical vein endothelial cells
MMP-9	matrix metalloproteinase 9
NHDFs	normal human dermal fibroblasts
PTK	protein tyrosine kinase
ROS	reactive oxygen species
RT	room temperature
TIMP-2	tissue inhibitor of metalloproteinase 2
VEGF	vascular endothelial growth factor

pose of this study was aimed at investigating whether genistein exhibited bivalent effects on ECs. For this, a wide range of concentrations was used to investigate its effect in 2D and in a 3D *in vitro* angiogenesis model. Results provided evidence that genistein bivalently modulated this process through acting via multiple pathways.

Results

Genistein, an isoflavone found in soy products (► Fig. 1), has been previously shown to affect endothelial capillary formation in 3D fibrin matrix assays in which dermal fibroblasts were used as a feeder layer. In the present study, a 3D angiogenesis *in vitro* model was used and genistein exhibited a bivalent effect on tube formation. Low concentrations of genistein (0.001–1 μM) increased pseudocapillary formation, whereas higher concentrations (25–100 μM) inhibited pseudo-microvessel outgrowth when compared to the vehicle control (0.01% DMSO) (► Fig. 2A). The effect of genistein on sprouting angiogenesis was assessed by using software that recognizes angiogenic processes. The recognition patterns utilized by the software are depicted in ► Fig. 2B. The effects of genistein on the total outgrowth network (total length), number of capillaries (anchorage junctions, extremities), and complexity of the network (branching interval, pieces, and branches) were quantified. Representative results indicated that genistein significantly promoted tube formation at low concentrations (0.01 μM), as revealed by total length and anchorage junctions (► Fig. 2C). In contrast, high genistein concentrations reduced tube formation starting around 25 μM , with statistical significance at concentrations of 50 μM and higher. Results obtained by measuring the other parameters are presented in Fig. 1S, Supporting Information.

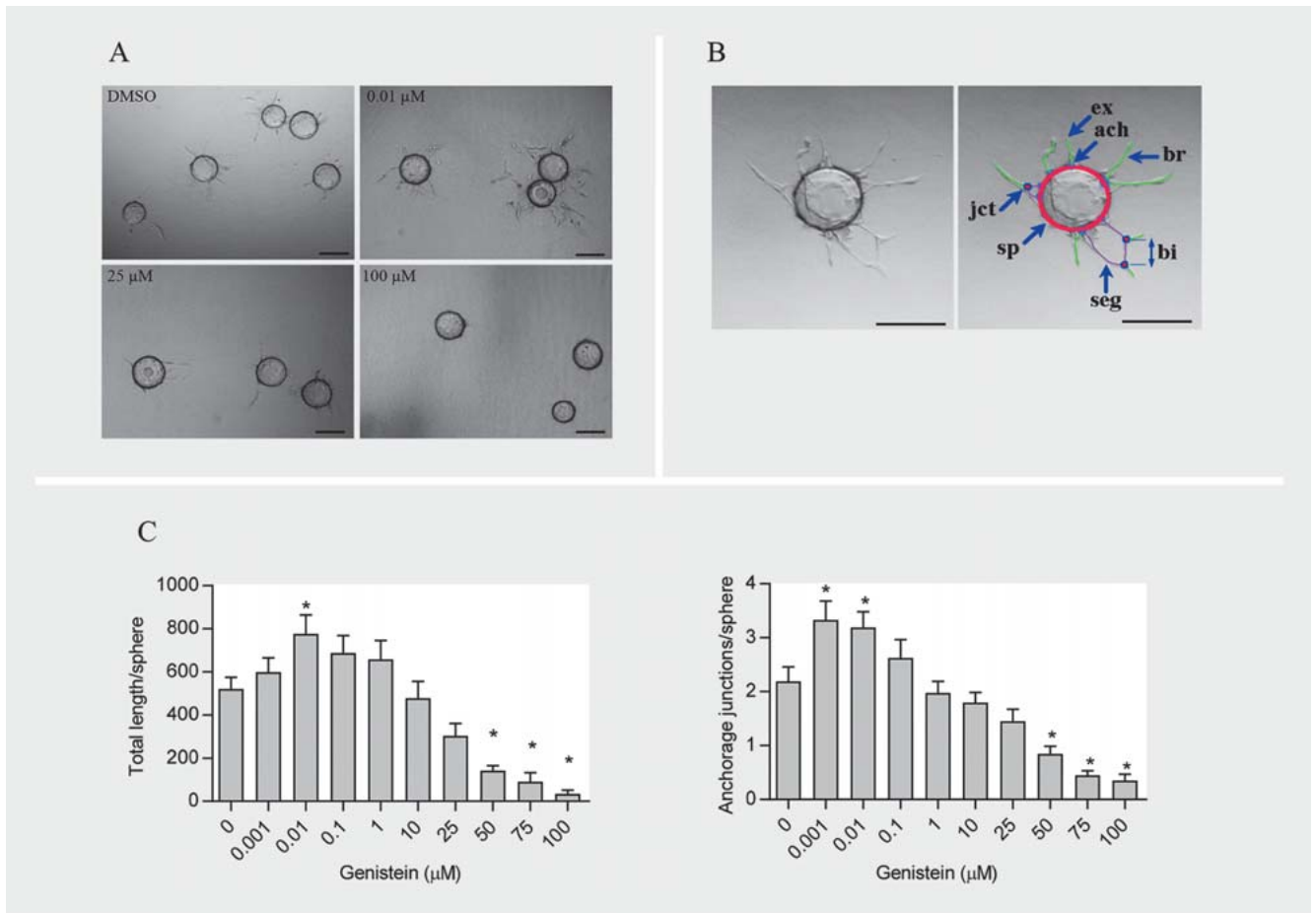


► Fig. 1 Chemical structure of genistein.

The main biological activities needed for activated HUVECs to form microcapillaries, including cell growth, metabolic activity, cell proliferation, and cell cycle distribution, were evaluated. In response to a 48-h genistein treatment, cell growth, metabolic activity, and proliferation as measured by MTT, CAM, and VPD450, respectively, were higher at low concentrations (0.001–0.01 μM), and significantly lower at higher concentrations (50–100 μM) (► Fig. 3A–C). In response to 24 h and 72 h genistein treatments, similar trends were observed for cell growth and metabolic activity (data not shown). After 8 h genistein treatment, HUVEC migration, measured by the wound healing assay, showed similar results (► Fig. 3D).

Endothelial cell migration has been partly recognized as being modulated by CD31-mediated cell-cell interactions. CD31 has been described as a factor regulating the assembly of junctional complexes required for the stable association of adjacent ECs in the wall of the vessel [12]. Therefore, cellular CD31 localization was investigated after genistein treatment. As revealed by immunocytochemistry, a 24-h treatment of HUVECs with low genistein concentrations (0.001–10 μM) resulted in an accumulation of CD31 to the cell membrane when compared to the vehicle control (► Fig. 4A). However, high genistein concentrations resulted in a CD31 decrease in cell-cell boundaries. Evaluation of CD31 protein expression by Western blotting under genistein stimulation (0.01, 25, and 50 μM) showed levels of expression equivalent to that of the vehicle control (► Fig. 4B), indicating that genistein affects CD31 localization and not expression.

By carefully examining the endothelial cells in the presence of high genistein concentrations, remarkable morphological alterations in HUVECs were evidenced. HUVECs treated for 48 h with genistein up to 10 μM displayed a typical F-actin organization with stress fibers running through the cytosol (► Fig. 5A) when stained with phalloidin. Higher concentrations (25–100 μM) resulted in reduced cell-cell contacts, and HUVECs phenocopied a myofibroblast morphology with an irregular, elongated shape. At 50 μM , the formation of lamellipodia was shown (► Fig. 5A). A morphometric analysis shown in ► Fig. 5B demonstrates the morphological change from cobblestone morphology to spindle shaped, evidenced by the measurement of circularity of the isolated cells, which was significantly reduced starting with 25 μM genistein. To test if the morphological changes were related to the conversion of ECs into myofibroblasts, markers of endothelial (VE-cadherin) and fibrotic characteristics ($\alpha\text{-SMA}$ and FSP-1) were examined. HUVECs treated with genistein did not show a decrease in the endothelial marker nor an increase in the fibrotic markers (► Fig. 5C).



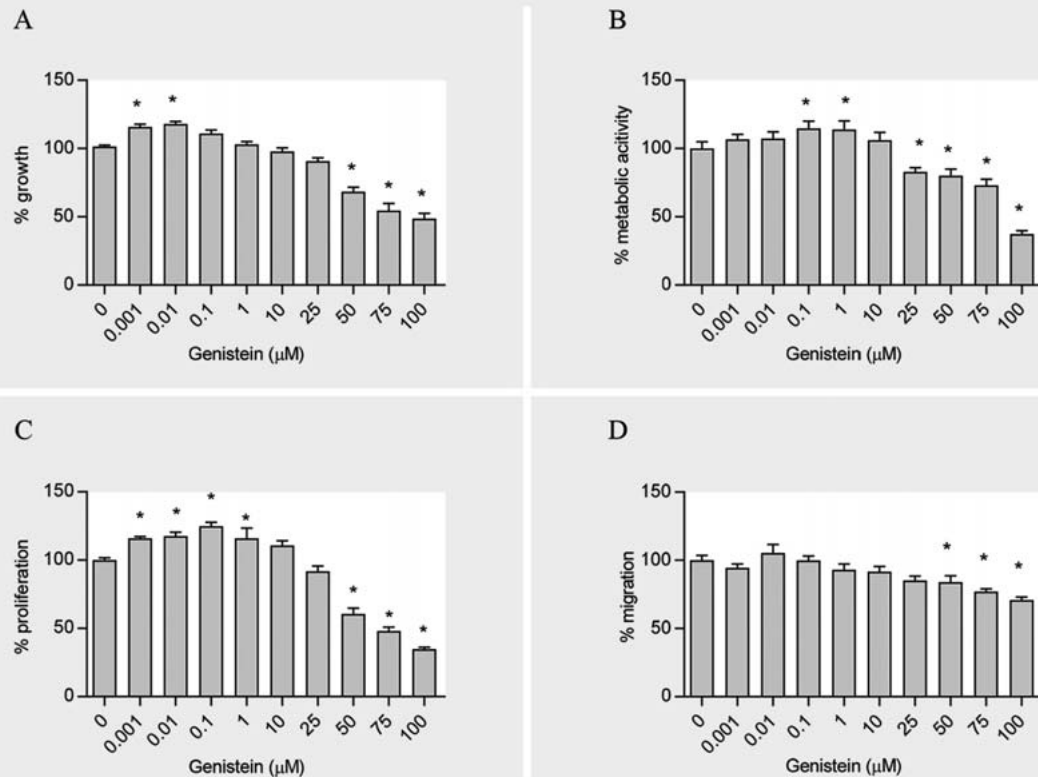
► **Fig. 2** Genistein bivalently modulated angiogenesis in a 3D fibrin gel. **A** Cells were treated with vehicle control or genistein (0.001–100 μM) for 4 days. Representative pictures of angiogenesis enhancement (genistein 0.01 μM) or inhibition (25 and 100 μM) compared to control conditions (DMSO) at day 4. Scale bar: 150 μm. **B** Cytodex microcarrier bead coated with HUVECs exhibiting pseudocapillary growth, before and after automatic analysis; ex: extremity, ach: anchorage junction, br: branche, jct: junction, sp: sphere, seg: segment, bi: branching interval. Scale bar: 150 μm. **C** Quantification of morphometrical parameters of the capillary network was performed by a computerized method on pictures taken on day 4. Representative parameters measured were total length and number of anchorage junctions per sphere. Graphs are representative of three independent experiments. One hundred spheres were quantified for each experimental condition. *Significantly different from vehicle control as measured by one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$).

To study the cellular effectors orchestrating the angiogenesis modulation upon a 24-h treatment with a range of genistein concentrations (0.001–100 μM), their mRNA expression was measured. Genistein downregulated the expression of *Notch4* mRNA levels and its target genes *PBX1* and *PPAR γ* . *VEGFA*, *SMAD3*, and *ANGPT-2* mRNA were significantly upregulated in response to genistein treatment (► **Fig. 6**).

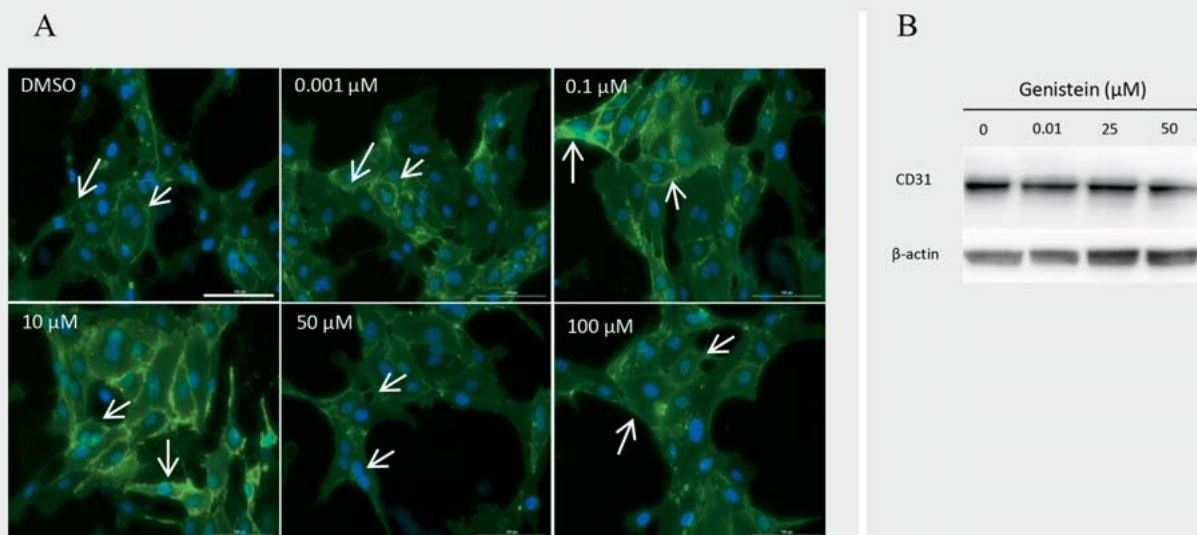
To determine if specific angiogenesis signature protein secretion was affected by genistein, an angiogenesis array was performed (► **Fig. 7**). Changes in secretion of potent angiogenic factors (bFGF, EGF, angiogenin, ANGPT-2), matrix metalloprotease and a tissue inhibitor of metalloproteases (MMP-9 and TIMP-2), a plasminogen activation system (also known as uPAR), and adhesion molecules (CD31) were observed in response to genistein (► **Fig. 7**).

Discussion

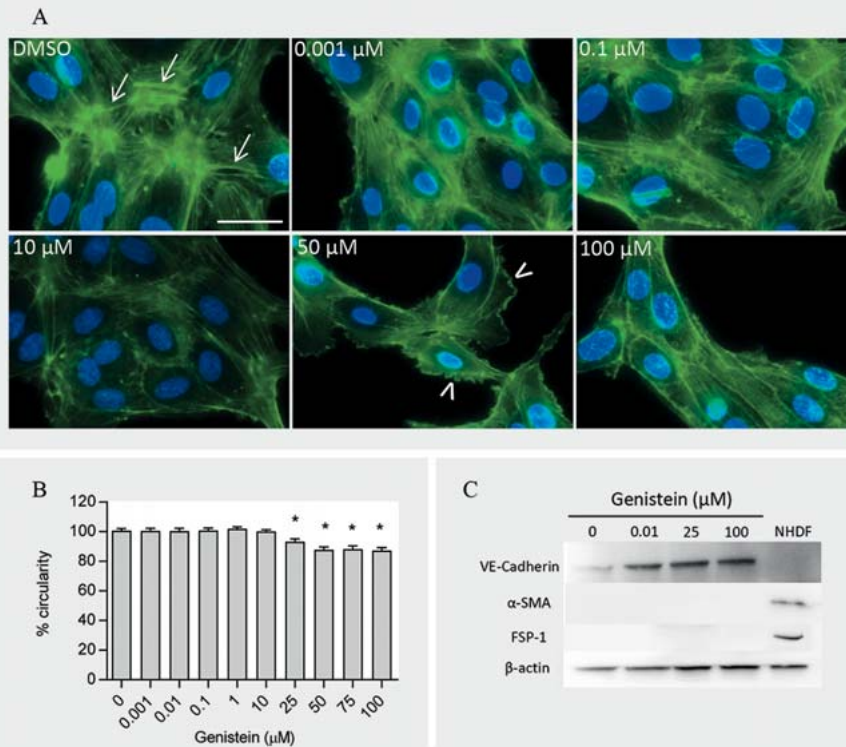
Genistein is an isoflavone used as a dietary supplement for its health promoting effects against cardiovascular diseases and cancers [13–15]. Genistein is currently considered a cancer chemopreventive drug candidate, and initial epidemiological studies in Asian populations consuming soy-rich diets showed a lower hormonal cancer incidence [16]. A typical Japanese soy-based diet contains 25–100 mg soy isoflavones/day, and estimates of daily genistein consumption delivered by soy-based diets are 0.25–1.0 mg/kg. In this population, the average plasma concentration of genistein was between 0.3 and 0.6 μM [17]. Clinical studies evaluating the effect of isoflavone/soy supplementation reported a wide range of genistein plasma concentrations in humans, with up to 40 μM for some of them [18]. However, most of the studies reported genistein concentrations ranging between 2 and 10 μM.



► **Fig. 3** Genistein bivalently regulated HUVEC growth, metabolic activity, proliferation, and migration. HUVECs were exposed to increasing concentrations of genistein (0.001–100 μM) for 48 h. The percentage of growth was measured using the MTT assay (A), metabolic activity was evaluated using CAM staining (B), and the proliferation rate was assessed using VPD 450 staining (C). D Effect of 8 h treatment with increasing concentrations of genistein on HUVEC migration in a wound healing assay. For all experiments, values represent the mean ± SEM of at least three independent measurements. * Significantly different from the vehicle control as measured by one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$).



► **Fig. 4** Effect of genistein on HUVEC CD31 expression and localization. A HUVECs were incubated with increasing concentrations of genistein (0.001–100 μM) for 24 h to determine the cellular distribution of CD31. CD31 expression was observed by immunocytochemistry (white arrows, A) and Western blot (B). Scale bar: 100 μm.



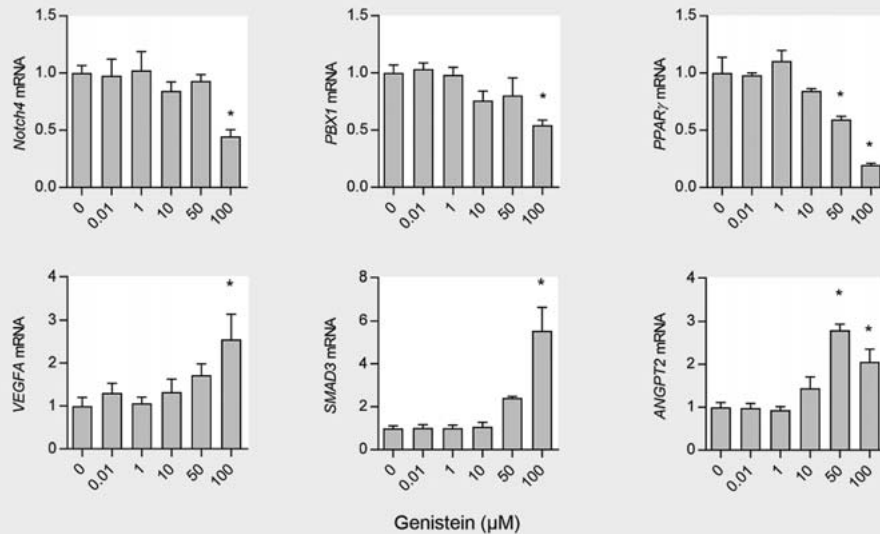
► **Fig. 5** Effect of genistein on HUVEC cytoskeleton organization and phenotypic conversion. **A** HUVECs were treated with increasing concentrations of genistein for 48 h. Analysis of actin filament distribution in HUVECs in response to genistein was detected using FITC-conjugated phalloidin, as described in Materials and Methods. Scale bar: 50 μm . Arrows show stress fiber formation; arrowheads show lamellipodia formation. **B** Cell circularity automatically quantified in the entire well, as described in Materials and Methods. **C** HUVECs were exposed to genistein (0.01, 25, and 100 μM) for 24 h, and protein expression was analyzed by Western blot in comparison to non-treated NHDFs. Values represent the mean \pm SEM of at least three independent measurements. * Significantly different from the vehicle control as measured by one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$).

With the growing number of studies addressing the health benefits of genistein, conflicting data arose. Some of them indicated that genistein may inhibit angiogenesis, and others claimed that it may be pro-angiogenic.

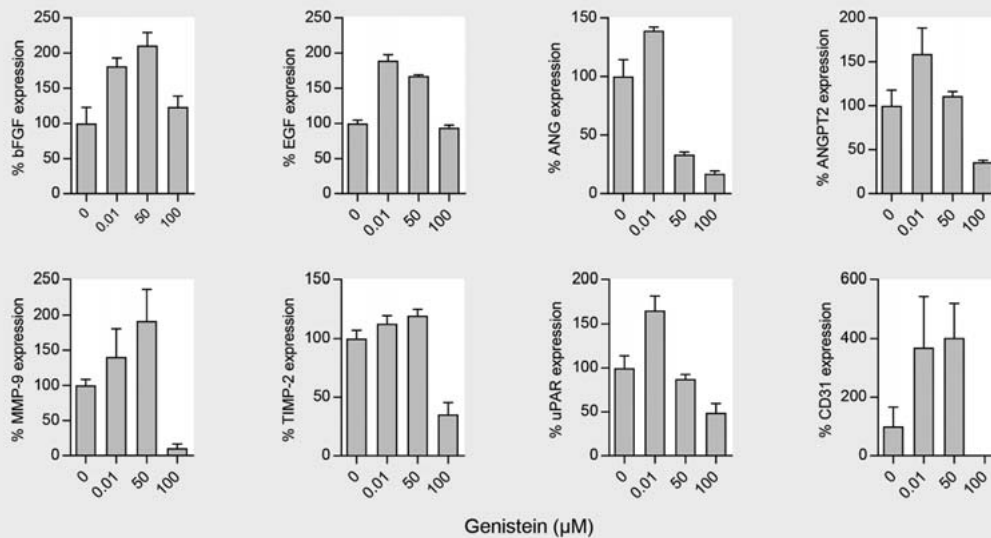
In the present study, a wide range of genistein concentrations were used, and a previously unreported bivalent effect, which consisted in an angiogenic stimulation of endothelial tube formation at low concentrations and angiogenic inhibition at higher concentrations, was observed. Low amounts of genistein significantly increased HUVEC angiogenesis, growth, metabolism, and proliferation, suggesting that it may play a role in stimulating cell physiology. However, a 200-fold increase in genistein concentration completely abrogated endothelial activities. In alignment with these observations, previous studies showed pro-angiogenic effects of genistein, where EPCs contributed to the process of neovascularization and tissue repair following ischemic myocardial events in animal models and in clinical trials. The effect of genistein on the engraftment of transplanted EPCs in an acute myocardial ischemic model was investigated [10], and the results indicated that genistein improved the migration and proliferation of EPCs. Genistein-treated EPCs resulted in the secretion of cytokines that promoted neovascularization, decreased myocardial fibrosis, and improved

cardiac function [10]. On the other hand, several studies have shown that genistein exhibited its antitumor effects by inhibiting angiogenic processes *in vitro* through a decrease in EC proliferation and angiogenesis at half-maximal concentrations of 5 and 150 μM , respectively [19]. Genistein was also evaluated for its *in vivo* antiangiogenic activities in nude mice xenograft and chick chorioallantoic membrane models. The compound exhibited a dose-dependent inhibition expression/excretion of the angiogenic factors VEGF, platelet-derived growth factor (PDGF), and the matrix-degrading enzymes urokinase-type plasminogen activator, MMP-2, and MMP-9 [20]. Additionally, an upregulation of angiogenesis inhibitors, such as plasminogen activator inhibitor-1, endostatin, angiostatin, and thrombospondin-1, was observed [20].

In the present study, further results showed a clear change in HUVEC morphology at high genistein concentrations. This morphological change was investigated to see if it was the signature of EndMT, a process where the endothelial cells transdifferentiate into mesenchymal cells. For this, CD31 cell localization was identified upon genistein treatments and it showed an effect on CD31 cell membrane localization. CD31 has been described as an angiogenic factor regulating the assembly of junctional complexes required for the stable association of adjacent ECs in the vessel



► **Fig. 6** mRNA level of genes implicated in the angiogenic process of HUVECs upon genistein treatment for 24 h. Values represent the mean \pm SEM of at least three independent measurements. * Significantly different from the vehicle control as measured by one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$).



► **Fig. 7** Effect of genistein on HUVEC secretome. Conditioned media derived from HUVECs treated with genistein (0.01, 50, and 100 μ M) for 48 h evaluated in the human angiogenesis antibody array. Relative protein levels of bFGF (basic fibroblast growth factor), EGF (epidermal growth factor), ANG (angiogenin), ANGPT-2 (angiopoietin-2), MMP-9 (matrix metalloproteinase 9), TIMP-2 (tissue inhibitor of metalloproteinase-2), uPAR (urokinase receptor), and CD31 (cluster of differentiation 31, also known as platelet endothelial cell adhesion molecule-1) are shown. Values represent the mean \pm SEM of two replicates.

wall [21]. This dose-dependent change in CD31 localization at the cell surface without affecting CD31 total protein expression was likely due to a redistribution of the protein pathway, which might affect endothelial morphology, and is correlated with the bivalent effect of genistein on the angiogenic process.

Microtubules functionally interact with the actin cytoskeleton and together they organize the cytoskeleton architecture, facilitating endothelial cell adhesion, motility, and growth, the necessary components of an angiogenic response. To evidence that the morphological change was associated with an elongation of the

cells, actin stress fibers were detected by immunofluorescence with FITC phalloidin. Interestingly, genistein stimulated F-actin re-arrangement at low concentrations (0.001–0.1 μ M), therefore promoting stress fiber formation that was no longer present at high concentrations (10–100 μ M). This is in line with the morphological change observed, as evidenced by cell circularity measurements that supported cell elongation upon genistein treatment at high concentrations. Endothelial (VE-cadherin) and fibroblastic phenotypic markers (α -SMA, FSP-1) further confirmed that the morphological changes accompanied with intracellular CD31 and F-actin reorganization did not relate to an EndMT. In alignment with these results, others have shown that several flavonoids, such as quercetin, kampferol, morin, fisetin, and apigenin, were morphologically active on HUVECs [22]. The authors argued that the observed morphological effects at low concentrations on HUVECs may underlie their angiogenic and vascular modulating properties.

Previous studies have shown that genistein selectively bound to estrogen receptor β and exerted various biological activities. In preclinical studies, genistein inhibited the growth of several types of cancer cells via various pathways, including topoisomerase and PTK inhibitions, decreased ROS production, and inhibited angiogenic transforming growth factor- β and EGF signaling pathways [23–26]. Therefore, it was suggested as a powerful pharmacological agent that may reduce the incidence of tumors or other angiogenic diseases such as rheumatoid arthritis, psoriasis, and diabetic retinopathy [27]. In addition, genistein downregulated Notch expression in pancreatic cancer cells, an effect that was linked to cell growth inhibition and apoptosis induction [28]. In alignment with these findings, the present study showed that at high concentrations, genistein downregulated *Notch 4* expression and its downstream targets *PBX1* and *PPAR γ* . However, it was unexpected that mRNA expression of the angiogenic factors *VEGFA*, *SMAD3*, and *angiopoietin 2* was upregulated in response to high genistein concentrations. This observation could be explained as a compensatory attempt in response to angiogenic inhibitory effects in normal ECs. Moreover, it has been shown that there are differences between paracrine and autocrine VEGF, which helps in maintaining cell homeostasis in normal tissue [18, 29]. Therefore, the *VEGFA* mRNA increase observed in HUVEC cells may reflect an upregulation of the autocrine form, and may be different from what would happen in tumoral conditions. In addition, protein secretion of bFGF, EGF, ANG, ANGPT2, MMP-9, TIMP-2, uPAR, and CD31 was upregulated in HUVECs treated with low genistein concentrations. At higher concentrations, protein secretion returned to the basal level or was downregulated compared to control-treated cells, thus confirming the bivalent effects of genistein when looking at angiogenic factor secretion. In conclusion, for the first time, this study presented the bivalent effects of genistein on angiogenesis in a 3D *in vitro* model and gave insights into the underlying cellular mechanisms. In regards to this and genistein bioavailability, its benefit for cancer chemoprevention, cancer treatment, or pro-angiogenic therapies has to be carefully considered.

Material and Methods

Chemicals

Genistein (99% purity) was purchased from Acros Organics. MTT and CAM were purchased from Sigma-Aldrich. Phalloidin and Fix & Perm solutions were purchased from Life Technologies.

Primary cell culture

HUVECs (PromoCell) were cultured in EBM-2 (PromoCell) supplemented with 2% Supplement Mix (PromoCell), thus constituting the ECGM-2. In all experiments, cells between passages 2 and 7 were used. NHDFs from juvenile foreskin (PromoCell) were grown in complete FGM (PromoCell) supplemented with 5 μ g/mL insulin and 1 ng/mL human basic fibroblast growth factor (PromoCell). NHDFs were used between passages 2 and 9. All cells were cultured in 5% CO₂ at 37°C, and media were replaced every 2 days.

Fibrin bead assay

Angiogenesis was evaluated by using a modified fibrin gel bead assay as previously described [30, 31]. This assay was carried out to analyze the 3D sprouting of HUVECs from the surface of dextran-coated Cytodex 3 microcarrier beads (GE Healthcare) embedded in fibrin gels. Dry beads were hydrated and autoclaved. HUVECs were mixed with beads at a cell density of 400 HUVECs per bead in a solution of 2500 beads per 1 mL ECGM-2 medium. Beads and HUVECs were cocultured at 37°C and 5% CO₂, and gently shaken every 20 min for 4 h to allow cell adherence to the bead surface. After this step, beads coated with cells were transferred to a 75-cm² tissue culture flask and incubated for 24 h. Then, HUVEC-coated beads were washed three times with 1 mL of ECGM-2 to remove non-coated cells and were suspended at a density of 1000 HUVEC-coated beads/mL in a solution of fibrinogen type I (2.5 mg/mL) with 0.15 U/mL of aprotinin at pH 7.4. Four μ L of 10 units/mL of thrombin were added into a 96-well optical plate (Corning). Eighty μ L of fibrinogen type I-protinin-HUVEC-coated beads solution were placed over the thrombin drops, gently mixed, and allowed to clot for 2 min at RT, and then at 37°C and 5% CO₂ for 30 min to promote gel formation. Eighty μ L of ECGM-2 medium were added to each well and equilibrated with the bead-containing gels for 30 min at 37°C and 5% CO₂. Afterwards, 3700 NHDFs were added on top of the gels and allowed to adhere. After 1 h, ECGM-2 was replaced with fresh medium with or without the test compounds. The medium was changed the day after and then every 2 days. Sprouting was apparent between days 2 and 3, and cultures were imaged on day 4. For the quantification of microvessel network sprouting, samples were automatically scanned with a high-throughput imager (IXM, Molecular Device) at 4x magnification at four sites of the wells in order to cover the entire surface of the wells [32]. Microsphere images analysis was performed using a plugin programmed for ImageJ software. This development is based upon the Angiogenesis Analyzer for ImageJ plugin. The inner areas of spheres were detected by a threshold using the so-called IsoData method after appropriate image pretreatments to correct lighting defaults and optimize sphere edge visualization. Light field correction and noise removal were performed by using a synthetic

flat field and fast Fourier transform band-pass filtering to identify pseudo-vascular trees. A gradient filter was then applied before segmentation with the so-called “percentile thresholding method”. Anchorage junctions between pseudocapillaries and sphere edges were detected together with junctions, extremities, segments, and branches. Segments were defined as lines ended by two junctions or by one junction and one anchorage, and branches as lines linked to one junction and one extremity or one anchorage and an extremity.

Cell growth, metabolic activity, proliferation and migration assays

MTT cell growth assay

HUVECs were seeded in 96-well plates at a density of 10^4 cells per well and incubated overnight in EBM-2 medium containing 0.5% FBS. The following day, cell cultures were treated for 24, 48, and 72 h with a range of genistein concentrations (0.001–100 μM) and then 10 μL MTT were added (final concentration 500 $\mu\text{g}/\text{mL}$) for 4 h at 37 °C. Genistein was dissolved in DMSO with a final solvent concentration of 0.01%. The medium was then aspirated, and insoluble formazan crystals were dissolved with 100 μL DMSO per well. Absorbance was read at 595 nm on a microplate reader (Biotek).

Calcein AM metabolic activity assay

HUVECs were seeded in black clear bottom 96-well plates (μClear , Greiner) at a density of 10^4 cells per well and allowed to adhere for 24 h under standard conditions. Cells were then treated with a range of genistein concentrations (0.001–100 μM) for 24, 48, and 72 h. Then, cells were washed twice with PBS and 100 μL of 1 μM CAM in PBS was added to the cells for 30 min at 37 °C. Fluorescence (excitation 490 nm and emission 520 nm) was then read and quantified using the Cytation 3 cell imaging multi-mode reader (BioTek).

Cell proliferation assay

HUVECs were seeded in 6-well plates at a density of 1.2×10^5 cells per well and cultured in EBM-2 medium containing 0.5% FBS. The following day, they were treated with a range of genistein concentrations (0.001–100 μM) for 24 and 48 h. Cell proliferation was assessed using VPD 450 proliferation dye (BD Biosciences). Quantification of cell proliferation was performed by flow cytometry using the Attune Acoustic Focusing Cytometer (Life Technologies) according to the manufacturer’s protocol.

Wound healing assay

HUVECs were seeded in 96-well plates at a density of 4×10^4 cells per well and grown to confluence for 24 h. A scratch was created in the HUVEC monolayer using a 10- μL pipette tip. The wells were washed twice with PBS to remove detached cells and pictures were taken in the center of each well. HUVECs were then incubated for 8 h in serum-free medium with a range of genistein concentrations (0.001–100 μM). After incubation, HUVECs were washed with PBS and fixed in 4% paraformaldehyde. One image per well (center position, same as time 0) was obtained on a high-throughput Cytation 3 cell imaging multi-mode reader

(BioTek). Quantification was performed using ImageJ software by measurements of the wounded areas at time 0 and 8 h.

Immunocytochemistry

HUVECs were seeded in black 96-well plates (μClear) at a density of 4×10^4 cells per well. Following a 24-h treatment with a range of genistein concentrations (0.001–100 μM), cells were washed twice with PBS, fixed with 4% paraformaldehyde for 30 min, and permeabilized with 0.1% Triton X-100 in PBS for 30 min at RT. They were then incubated in blocking buffer (5% milk in PBS) for 2 h at RT, washed with PBS, and incubated with primary monoclonal antibody mouse anti-human CD31 (Abcam, 1:50) for 2 h at RT. After incubation, cells were washed twice with PBS and incubated with the secondary antibody goat anti-mouse IgG H&L Dylight® 488 (Abcam, 1:200) for 1 h at RT. Nuclei were counterstained with DAPI (Life Technologies, 1:1000).

Western blotting analysis

HUVECs were seeded in 6-well plates at a density of 3×10^5 cells per well and treated with genistein (0.01, 1, and 100 μM) for 24 h. Then, they were lysed in ice-cold cell lysis buffer (Cell Signaling), and the protein concentration was determined using the Bradford method. Twenty micrograms of proteins were subjected to 12.5% SDS-PAGE, and resolved proteins were transferred into a PVDF membrane. Membranes were blocked with 5% milk for 2 h and then probed with specific primary antibodies CD31 (Abcam, 1:1000), $\alpha\text{-SMA}$ (kind gift from Dr. Olivier Dorchies, 1:400), FSP-1 (Abcam, 1:100), VE-cadherin (Santa Cruz Biotechnology, 1:200), and $\beta\text{-actin}$ (Sigma, 1:3000), and prepared according to the manufacturer’s instructions. Protein loading was controlled by $\beta\text{-actin}$ immunoblotting. Bands were visualized by enhanced chemiluminescence detection (Amersham).

Cell morphology assessment

Cells were seeded in black with clear bottom 96-well plates (μClear , Greiner) at a concentration of 10^5 cells/mL in EBM-2 supplemented with 0.5% FBS for 24 h, and then treated with a range of genistein concentrations (0.001–100 μM) for 48 h. HUVECs were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 5 min at RT. HUVECs were then stained with 50 μL of 5 U/mL phalloidin (Life Technologies), washed twice with PBS, and marked with 50 μL of 1 $\mu\text{g}/\text{mL}$ DAPI for 5 min. HUVECs were visualized using the Cytation 3 cell imaging multi-mode reader (BioTek). Cell circularity was quantified by Gen 2.0 software using the average of 12 pictures of each well.

Quantitative real-time PCR

HUVECs were seeded in 12-well plates at a density of 3×10^4 cells per well and allowed to adhere for 24 h under standard conditions. They were then treated with a range of genistein concentrations (0, 0.1, 1, 10, 50, and 100 μM) for 24 h. Cells were lysed and total RNA was isolated from lysates using the Aurum total RNA Mini Kit (Bio-Rad) according to the manufacturer’s instructions. RNA (0.5 μg) was then reverse-transcribed using High-Capacity Total RNA-to-cDNA Reverse Transcriptase (Life Technologies) and a PCR standard thermal cycler (Life Technologies). One μL of the cDNA product was amplified by quantitative PCR using 125 nM

gene-specific primers in a total volume of 20 μ L using a SYBR Green PCR Kit (Life Technologies) and a Step One Plus Real-Time PCR Thermal Cycler (Life Technologies). Relative gene expression of *Notch4*, *PBX1*, *PPAR γ* , *VEGFA*, *SMAD3*, and *angiopoietin 2* (*ANGPT-2*) was normalized to the average of *GAPDH*, *cyclophyllin A*, and *β -actin* using the $\Delta\Delta$ CT method.

Angiogenesis protein array

RayBio Human Angiogenesis Antibody Array C-1000 (RayBiotech) was used to assay the conditioned medium. To prepare the conditioned medium, HUVECs were seeded in T25 flasks at a concentration of 5×10^5 cells per flask in ECGM-2. The following day, the medium was replaced with EBM-2 and 0.5% FBS, and the cells were treated with genistein (0.01, 50, and 100 μ M). After 48 h, the medium was collected and centrifuged at 400 g for 5 min to remove cellular debris. The supernatant was collected and frozen at -80°C until further use. In the array, 43 angiogenic growth factors were measured according to the manufacturer's protocol. Briefly, changes in the secreted angiogenic factors in response to various concentrations of genistein were compared. Membranes were imaged using a fusion system (Vilber Lourmat). Protein levels were quantified using the Protein Array Analyzer in the ImageJ program [33].

Statistical analysis

For each cell culture experiment, treatments were performed in triplicate or quadruplicate. Unless otherwise stated, each experiment was repeated three times. Data are expressed as means \pm SEM. One-way ANOVA was used for multiple comparisons in experiments with one independent variable. A Dunnett's test was used for post hoc analysis of the significant ANOVA. A difference in mean values between groups was considered to be significant when $p \leq 0.05$.

Supporting information

Other morphometrical angiogenesis parameters obtained through automatic quantification are available as Supporting Information.

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Conflict of Interest

The authors declare no conflicts of interest.

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