

Lawrence Berkeley National Laboratory

Biological Systems & Engineering

Title

[10]-Gingerol Reverts Malignant Phenotype of Breast Cancer Cells in 3D Culture

Permalink

<https://escholarship.org/uc/item/04n87327>

Journal

Journal of Cellular Biochemistry, 118(9)

ISSN

0730-2312

Authors

Fuzer, Angelina M
Lee, Sun-Young
Mott, Joni D
et al.

Publication Date

2017-09-01

DOI

10.1002/jcb.25906

Peer reviewed

FREE Webinar:

ClearLLab LS: A New CE-IVD Orientation Tube to Guide You Through Diagnosis of Hematolymphoid Malignancies

Dr. Katherina Psarra from the Immunology Dept. at Evangelismos Hospital, Athens and Dr. Michael Kapinsky, one of Beckman Coulter's clinical flow cytometry experts, will present:

- Determination of immunologically defined leukocyte subpopulations
- Immunophenotyping of hematological malignancies
- Influence of instrument and dye performance characteristics on your phenotyping results
- Panel design driven by: dye brightness vs antigen density; spillover-spreading and co-expression patterns

Thursday | July 12, 2018
3pm CEST | 9 am EDT

**Receive a FREE
eCasebook when
you Register Here**

[10]-Gingerol Reverts Malignant Phenotype of Breast Cancer Cells in 3D Culture

Angelina M. Fuzer,¹ Sun-Young Lee,² Joni D. Mott,² and Marcia R. Cominetti ^{1*}

¹Department of Gerontology, Federal University of São Carlos, São Carlos, SP, Brazil

²Biological Systems and Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, California

ABSTRACT

Breast cancer is a complex and multifactorial disease. Tumors have a heterogeneous microenvironment, which have multiple interactions with other cell types, greatly influencing the behavior of tumor cells and response to therapy. The 3D culture mimics the microenvironment better found in vivo and is more appropriated than the traditional 2D culture made from plastic to test the cellular response to drugs. To investigate the effects of [10]-gingerol on breast tumor cells, we used physiologically relevant three-dimensional (3D) cultures of malignant and non-malignant human breast cells grown in laminin-rich extracellular matrix gels (lr-ECM). Our results showed selective cytotoxicity of [10]-gingerol against the malignant T4-2 breast cancer cell line compared to non-malignant S1 cells. The compound reverted the malignant phenotype of the cancer cells, downregulating the expression of epidermal growth factor receptor (EGFR) and β 1-integrin. Moreover, [10]-gingerol induced apoptosis in this cell line. These results suggest that [10]-gingerol may be an effective compound to use as adjuvant therapy in breast cancer treatment. *J. Cell. Biochem.* 118: 2693–2699, 2017. © 2017 Wiley Periodicals, Inc.

KEY WORDS: 3D CULTURE; [10]-GINGEROL; BREAST CANCER; LAMININ-RICH EXTRACELLULAR MATRIX; MICROENVIRONMENT; NATURAL PRODUCTS

Ginger (*Zingiber officinale* Roscoe) is the rhizome of Zingiberaceae family plants, world known and used as a spice in cooking. Originally from Southeast Asia, ginger has been widely used as a medicinal plant for thousands of years, especially in its native region. Various studies describe their anti-inflammatory, -microbial, -oxidant, and -emetic activities [Haniadka et al., 2012]. Much of the ginger bioactivity is due to its phenolic compounds [4], [6], [8], and [10]-gingerols, paradols, and shogaols, which, particularly for gingerols, have an anti-proliferative and -angiogenic action on tumor cells, as demonstrated by several in vivo and in vitro studies [Liu et al., 2012]. Our group demonstrated that [10]-gingerol was more efficient, compared to [6] and [8]-gingerol, inhibiting cell proliferation of MDA-MB-231 breast cancer cells with more selectivity compared to non-tumor cells [Almada da Silva et al., 2012].

Breast cancer is a complex and multifactorial disease. Tumors have a heterogeneous microenvironment with multiple interactions with other cell types, such as macrophages, endothelial cells, and fibroblasts, as well as extracellular matrix (ECM) components. These interactions may involve autocrine, paracrine, and endocrine signals related to gene transcription, cell death, growth, differentiation, and

survival, which are altered during tumor progression [Weaver et al., 1996; Thoma et al., 2014; Weigelt et al., 2014].

Laminin-111 is one of the main constituents of basement membrane (BM) and plays a central role in maintaining the differentiation status and morphogenesis in breast tissue [Bissell et al., 2003]. When non-malignant human breast cells, such as HMT 3522 S1 cells, are grown in three-dimensional (3D) gels made of laminin-rich extracellular matrix (lr-ECM), they form growth arrested acinar-like structures that resemble those found in the human breast. Basal junctions in the acini mainly contain α 6/ β 4 integrins linked to BM laminin-332, basally secreted by S1 cells. Cell-cell junctions are characterized by side localized β -catenin/E-cadherin in adherent junctions and ZO-1/ZO-2 in lateroapical tight junctions. On the other hand, malignant breast cells, such as HMT 3522 T4-2 cells, form disorganized colonies that exhibit continuous growth and do not show apicobasal polarity [Petersen et al., 1992]. When in lr-ECM 3D culture, T4-2 cells form disorganized multicellular tumor-like masses with uninterrupted proliferation. Overexpression of epithelial growth factor receptor (EGFR) is one of the reasons that promote this continuous growth. Interactions between T4-2 cells and ECM are mediated by

Conflicts of interest: The authors declare that they have no conflicts of interest.

Grant sponsor: (FAPESP) São Paulo Research Foundation; Grant numbers: 2012/18908-6, 2013/00798-2, 2015/08146-0.

*Correspondence to: Marcia R. Cominetti, Universidade Federal de São Carlos, Rod. Washington Luis, Km 235, Monjolinho, São Carlos 13565-905, SP, Brazil. E-mail: mcominetti@ufscar.br

Manuscript Received: 5 December 2016; Manuscript Accepted: 20 January 2017

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 23 January 2017

DOI 10.1002/jcb.25906 • © 2017 Wiley Periodicals, Inc.

β 1-integrins, highly expressed on these cells compared to non-malignant S1 cells. β 1-integrins in T4-2 cells bind to laminin-111 from lr-ECM. However, it is possible to revert the malignant phenotype of these cells interfering in some pathways, such as blocking β 1-integrin or inhibiting EGFR or MAPK, re-establishing basoapical polarity, and obtaining acinar structures similar to non-malignant cells [Wang et al., 1998; Weaver et al., 2002; Vidi et al., 2013]. Similar to β 1-integrins, EGFR is also overexpressed on malignant T4-2 cells, leading to uninterrupted growth [Weaver and Bissell, 1999b]. However, their blocking and inhibition of MAPK kinase activation induces a decrease in the expression of both receptors [Wang et al., 1998; Weaver and Bissell, 1999b]. In a 3D system, as in vivo, there is a bidirectional cross-modulation between these MAPK and EGF signaling pathways that does not occur in 2D monolayers [Wang et al., 1998]. An attenuation of MAPK/EGF induced activation causes cell growth arrest [Wang et al., 1998].

It was demonstrated that the use of a function blocking antibody against β 1-integrin, AIIB2, was able to revert the malignant phenotype in HMT-3522 series T4-2 cells, as well as in MCF-7 and Hs58T breast cancer cells [Wang et al., 1998, 2002; Weaver and Bissell, 1999b]. Subsequently, cell polarization provided by β 4-integrin in 3D culture was related to the resistance of apoptosis in non-malignant breast cells and in breast malignant cells whose phenotype was reverted. The conversion was also observed when the polarization of normal cells was disrupted using blocking antibodies of E-cadherin [Weaver et al., 2002].

As the 3D cultures not only better mimic the microenvironment found in vivo, but also take into account the interaction of cells with the ECM components, we aimed to investigate the effects of [10]-gingerol on HMT-3522 progression series cells growing in lr-ECM in 3D culture. Results indicate that [10]-gingerol presents selective cytotoxicity toward the malignant T4-2 breast cancer cell line compared to non-malignant S1 cells. Moreover, [10]-gingerol was able to induce apoptosis and to revert the malignant phenotype of T4-2 cells.

MATERIALS AND METHODS

CELL LINES AND MEDIA

Human HMT3522 mammary epithelial progression series S1 (non-malignant) cells were originally isolated from a benign mammary fibrocystic nodule collected from a reduction mammoplasty tissue [Briand et al., 1987]. This cell line became immortalized spontaneously in culture and gave rise to the non-malignant S1 cell line, which is EGF dependent for growth. After continuous passages without EGF, a new non-malignant line arises. This cell line produced a rare tumor when injected into nude mice. The tumor cells were isolated in culture and reinjected into the mice originating the malignant T4-2 cell line, a triple-negative breast cancer cell line with an invasive phenotype [Schmeichel et al., 1998; Rizki et al., 2008; Vidi et al., 2013].

S1 and T4-2 cells were maintained in a chemically defined H14 medium [DMEM-F12 (Thermo Scientific) serum free-medium containing 250 ng/ml insulin (Thermo Scientific), 10 mg/ml

transferrin (Sigma-Aldrich), 2.6 ng/ml sodium selenite (Collaborative Research), 10 nM β -estradiol (Sigma-Aldrich), 1.4 μ M hydrocortisone (Collaborative Research), and 5 mg/ml prolactin (Sigma)] in a humidified incubator at 37°C and 5% CO₂. Non-malignant S1 cells were maintained in the presence of 10 ng/ml EGF (Collaborative Research). MDA-MB-231 cells (malignant) were purchased from ATCC (catalog number HTB-26) and were cultivated in 10% FBS DMEM medium containing 1% penicillin/streptomycin.

REAGENTS

[10]-gingerol was provided by the Natural Products Laboratory, Department of Chemistry, UFSCar using a purification method previously described [Almada da Silva et al., 2012]. PD98059 was purchased from EMD Millipore and AIIB2 from Aragen Bioscience. The latter is a rat monoclonal IgG and was purified from a hybridoma cell line.

THREE-DIMENSIONAL CELL CULTURE

Experiments in 3D culture were performed using a lr-ECM gel: Matrigel[®] (BD Biosciences). The 3D cell culture was carried out according to the protocol previously described by Lee et al. [2007] using the “on top” method. Briefly, 24- or 6-well-culture plates were coated with 0.5 ml of chilled Matrigel[®] spread with a pipette tip to avoid bubbles. Plates were incubated in a humidified incubator at 37°C and 5% CO₂ for 20–30 min for polymerization. Cells were trypsinized, counted, and seeded on the plate and chilled medium with 10% Matrigel was added. Different concentrations of [10]-gingerol were mixed with the chilled remaining medium containing Matrigel and added to cells. Plates were incubated for 3–4 days. The images were visualized and photos were taken using a Nikon Eclipse TS 100 microscopy under 10 \times magnification.

IMMUNOSTAINING

Cells were fixated with 4% paraformaldehyde solution for 20 min and washed three times with PBS-glycine (50 mM) for 10 min. Samples were blocked in immunofluorescence solution (IF) (1.3 M NaCl, 132 mM Na₂HPO₄, 34.5 mM NaH₂PO₄, 77 mM NaN₃, 1% BSA, 2% Triton-X-100, 0.5% Tween-20, pH 7.4) containing 10% goat serum and goat anti-mouse IgG F(ab¹)₂ (Santa Cruz Biotechnologies) for 1 h at room temperature. Subsequently, primary antibody staining was carried out using the following antibodies: anti- α 6-integrin (1:300), anti- β -catenin (1:50) (BD Biosciences), GM130 (D6B1) \times P(R) (1:1000), and anti-cleaved caspase 3 (Cell Signaling Technologies), overnight at 4°C. Samples were washed in IF for 20 min, three times. Alexa Fluor-488 and -568 (1:500) coupled to secondary antibodies were incubated for 1 h at room temperature. After washing the samples twice in PBS for 10 min, DAPI (diamidino-2-phenylindole) staining was performed using 0.5 ng/ml DAPI solution (Thermo Scientific) for 4 min at room temperature and samples were washed again 10 min in PBS before mounting the slides with Fluoromount G (Electron Microscopy Sciences). Samples were photographed under AxioVision Fluorescence Microscope (Zeiss Microscopy) at 40 \times magnification and analyzed using Image J Software.

CALCEIN-DAPI ASSAY AFTER STRUCTURE FORMATION

This assay was conducted after formation of the 3D structures, 6 days for S1 cell lineage and 4 days for T4-2 and MDA-MB-231 cells. Cells were treated with different concentrations of [10]-gingerol for 3 days. After treatment, structures were stained using 0.001 mg/ml calcein acetoxyethyl-diacetylester (calcein AM) (Invitrogen) and 0.05 mg/ml DAPI (Thermo Scientific) for 1 h inside the incubator (37°C, 5% CO₂). Images were captured onto AxioVision Fluorescence Inverted Microscope (Zeiss Microscopy) at 20× magnification and analyzed using Image J Software.

PROTEIN EXTRACTION

To carry out the experiments involving protein extraction, the ECM gels from the 3D cell culture were solubilized as described previously [Lee et al., 2007], using cold PBS-EDTA (5 mM) solution containing NaVO₄ (1 mM), NaF (1.5 mM), and protease inhibitor cocktail (EMD Millipore). The solution was transferred to tubes and gently stirred in a cold room (4°C) for 20 min to dissolve Matrigel[®] and centrifuged for 5 min at 800 rpm. Obtained pellets were lysed with RIPA buffer (150 mM NaCl; 50 mM Tris-HCl pH 7.4; 50 mM NaF; 2 mM EDTA; 1.0% NP-40; 0.5 mM Na-deoxycholate; 0.1% SDS, pH 8.0) and transferred to a 1.5 ml microtube, sonicated for approximately 20 s on ice and centrifuged for 20 min at 12,000 rpm.

IMMUNOBLOT ANALYSIS

Protein concentrations of supernatants were determined using a DC Protein Assay kit (Bio-Rad). Protein samples (15 µg) were applied onto a 4–20% Tris-glycine gel (Life Technologies), transferred to nitrocellulose membranes (GE Amersham), and incubated with anti-β1-integrin, -EGFR, and -β-catenin antibodies (BD Biosciences), followed by incubation with HRP-conjugated goat anti-mouse secondary antibody (Thermo Scientific). Beta-catenin was used as the loading control. Substrate development was performed using SuperSignal West Dura Extended Substrate reagent (Thermo Scientific). Specific bands were visualized with FluorChem imager (Alpha Innotech) and quantified with Image J software, normalized to β-catenin.

RESULTS

[10]-GINGEROL INDUCES PHENOTYPIC REVERSION OF MALIGNANT T4-2 CELLS IN 3D LR-ECM

To optimize [10]-gingerol concentrations in phenotypic reversion of malignant T4-2 cells in 3D culture, a morphology assay was performed. Two previously validated reverting agents, a mechanistic inhibitor of MAPK (PD98059) and β1-integrin function blocking antibody (AIIB2) were also tested to verify which one was the best to compare to [10]-gingerol. [10]-gingerol was also tested on the non-malignant S1 cells to determine selective cytotoxicity toward malignant cells. [10]-gingerol did not promote any significant change on the morphology of S1 cells (Fig. 1A).

On the other hand, malignant T4-2 cells treated with [10]-gingerol had a remarkable reduction on the 3D structure size, similar to that observed with PD98059 and AIIB2 antibody treatments, suggesting that this natural product has a potential to modulate the malignant

phenotype. It can be observed that 1 µM [10]-gingerol started to induce the reduction of the structure size (Fig. 1A). When treated with concentrations above 2.5 µM, some disaggregation was observed on the structures, which could indicate an occurrence of apoptosis (Fig. 1A). The effects of [10]-gingerol on a triple negative breast cancer cell line, MDA-MB-231, were also analyzed. When in 3D culture, MDA-MB-231 cells formed huge three-dimensional tumor-like structures. The cells showed an increased sensibility to [10]-gingerol, with a notable reduction of structure size in treatments at 1 µM and above (Fig. 1A), suggesting that [10]-gingerol effects are not limited to T4-2 cells, but extend to other human breast cancer cells.

It is well established that basoapical polarity confers apoptosis resistance in non-malignant S1 cells [Weaver et al., 2002]. However, in previous results, where cells were treated with [10]-gingerol at the same time they are plated, subtle differences were found in treated S1 morphology when compared to the controls. To better mimic the physiological conditions *in vivo*, when tumor cells are already established in the tumor prior to treatment, a cytotoxicity assay using calcein AM and DAPI double staining was carried out after full growth of the 3D culture structures (Fig. 1B). After the formation of 3D structures, 6 days for S1 cells and 4 days for T4-2 and MDA-MB-231, due to high proliferation rates of these malignant lines, cells were treated with different concentrations of [10]-gingerol for 3 days. Live cells were identified by an intense and uniform green fluorescence due to intracellular esterase activity, which enzymatically converts calcein AM to fluorescent calcein, retained by viable cells (Fig. 1B). On the other hand, cells that lost membrane integrity had the nuclear DNA stained with DAPI, which is excluded by viable cells that have an intact membrane. Results showed no significant differences in DAPI staining when treated with [10]-gingerol up to 10 µM in S1 cells. On the other hand, in the T4-2 and MDA-MB-231 pre-formed structures, cell death and, consequently, a reduction in colony size was observed compared to the vehicle used in the controls (Fig. 1B). These data indicate that [10]-gingerol could present selective cytotoxic for malignant cells with little or no damage to normal cells in physiological conditions.

To confirm that [10]-gingerol treatment phenotypically reverted the malignant phenotype of T4-2 cells, immunostaining was performed using anti-α6-integrin and -β-catenin antibodies to determine the polarity of the resulting T4-2 cell colonies (Fig. 1C). The results corroborated with the previous morphology assay. T4-2 3D structures were not only smaller, but also [10]-gingerol allowed T4-2 cells to form polarized normal-like structures in lr-ECM 3D culture, which exhibited a similar organization to S1 acinar organoids (Fig. 1C), as well as the reverted controls of T4-2 cells treated with PD98059 and AIIB2 (Fig. 1C). On the other hand, MDA-MB-231 treated cells had a reduction in colony size, but a reorganization into acinar organoids was not observed (Fig. 1C). This result is similar to previous reports where MDA-MB-231 cells were not phenotypically reverted when using only one reverting agent [Wang et al., 2002].

[10]-GINGEROL MODULATES THE LEVEL OF EGFR AND β1 INTEGRIN IN MALIGNANT T4-2 CELLS

T4-2 cells overexpress EGFR and β1-integrin adhesion receptors [Weaver and Bissell, 1999a]. When T4-2 cells are reverted in 3D

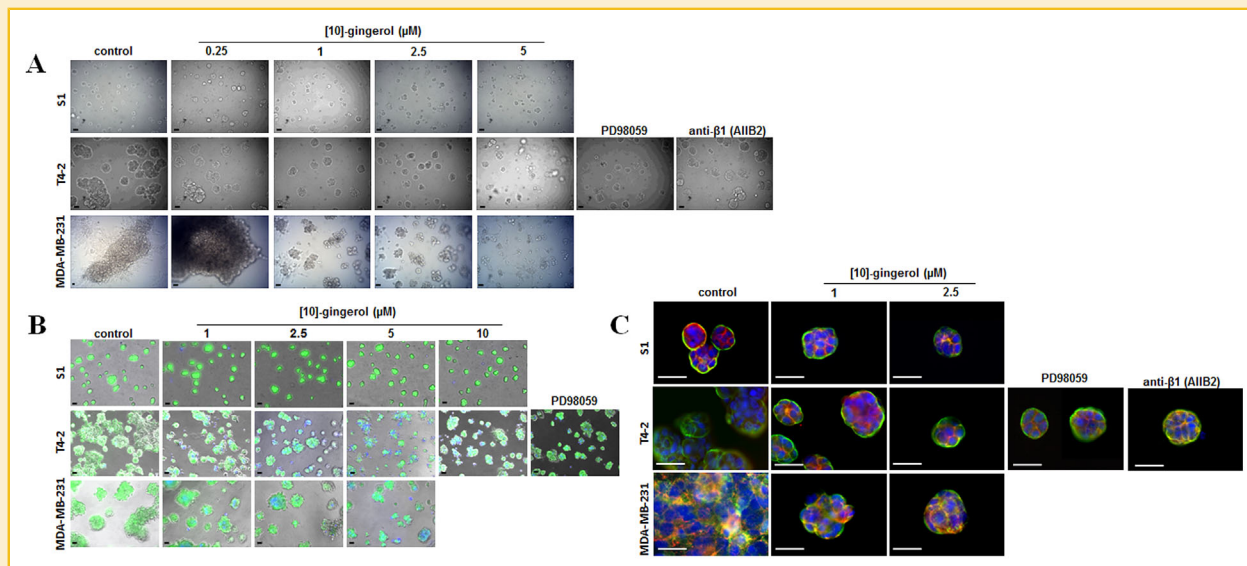


Fig. 1. Effects of [10]-gingerol treatment on the modulation of malignant phenotype and selective cytotoxicity on tumor cells. (A) Cells, previously incubated with mentioned concentrations of [10]-gingerol, PD98059, or AIB2, were seeded on the Matrigel[®] suitable medium and images were captured onto a Nikon Eclipse TS 100 microscopy under 10 \times magnification. (B) Calcein AM/DAPI live/dead assay double staining, conducted 6 (for S1 cells) or 4 (for T4-2 and MDA-MB-231 cells) days after the formation of 3D structures and treated with described concentrations of [10]-gingerol for 3 days. Living cells were identified by an intense and uniform green fluorescence color and dead cells by blue. (C) Immunostaining of non-malignant S1 and malignant T4-2 and MDA-MB-231 cells. Cells, previously incubated with mentioned concentrations of [10]-gingerol, PD98059, or AIB2, were seeded on the Matrigel[®] suitable medium. The 3D structures were collected from the wells in which they were grown, fixed with 4% paraformaldehyde, and stained with anti- β 1-integrin (AIB2 antibody, green), anti- β -catenin (red), and with the Alexa Fluor secondary antibody, for immunostaining procedures. Nuclei are stained with DAPI (blue). Images were taken using an AxioVision Fluorescence Inverted Microscope (Zeiss Microscopy) under 40 \times magnification (scale bars 40 μ m).

culture, there is a cross-modulation between these two signaling pathways and both of these proteins are down-modulated. Growth suppression observed on reverted T4-2 3D structures is correlated with an attenuation of MAPK/EGF induced activation, which could be an alternative therapeutic strategy against breast cancer in the early stages [Wang et al., 1998]. Inhibition of MAPK kinase activation and also inhibition of EGFR or β 1-integrin induce a decrease in the expression of both EGFR and β 1-integrin [Wang et al., 1998; Weaver and Bissell, 1999a].

To determine whether [10]-gingerol induces down-modulation of EGFR and β 1-integrin as seen in treatments with other reverting agents, immunoblotting was performed. Results showed that [10]-gingerol was able to induce a downregulation of both receptors, EGFR and β 1-integrin in T4-2 cells, explaining the occurrence of growth-arrest and restoration of basolateral polarity in 3D structures, as previously observed. The suppression by [10]-gingerol is comparable to treatment with the MAPK inhibitor, PD98059 used as the control. S1 cells, which express lower levels of EGFR and β 1-integrin, showed no significant alterations when treated with [10]-gingerol (Fig. 2).

As we previously observed that [10]-gingerol above 2.5 μ M showed morphological characteristics of apoptosis in malignant T4-2 cells, we further sought to determine whether [10]-gingerol could induce apoptosis in 3D Ir-ECM culture. Immunostaining using an anti-cleaved caspase-3 antibody, as expected, showed no presence of apoptotic cells on S1 cells treated with [10]-gingerol, similar to the control (2.5%) (Fig. 3A and D).

However, T4-2 reverted structures concentrated cleaved caspase-3 staining in the center of the structures, in the luminal compartment in 25% of cells (Fig. 3B and D). This phenomenon could be explained by the interactions of basally located cells with ECM, which protect them from apoptosis [Park et al., 2006; Haniadka et al., 2012]. MDA-MB-231 disorganized colonies were extensively positive for cleaved caspase-3, confirming previous results using 2D culture (manuscript in preparation) (Fig. 3C and D).

DISCUSSION

Within 3D Ir-ECM gels, non-malignant S1 cells form polarized, multi-cellular growth-arrested and acinus-like colonies in response to the laminin-111 in the BM, resembling the acinar structures of normal human mammary tissue. In contrast, under the same conditions, malignant T4-2 cells form disorganized and non-polar colonies that grow continuously [Lee et al., 2007; Rizki et al., 2008]. EGFR, integrin, and aerobic glycolysis-signaling pathways are hyper activated in malignant T4-2 cells and reciprocal interactions among these pathways occur in T4-2 cells when grown in 3D Ir-ECM [Weaver et al., 1997; Onodera et al., 2014].

It was demonstrated that the inhibition of various signaling pathways enables T4-2 cells to form polarized growth-arrested colonies (without any change in their genotype), presenting a morphology similar to the colonies formed by non-malignant S1

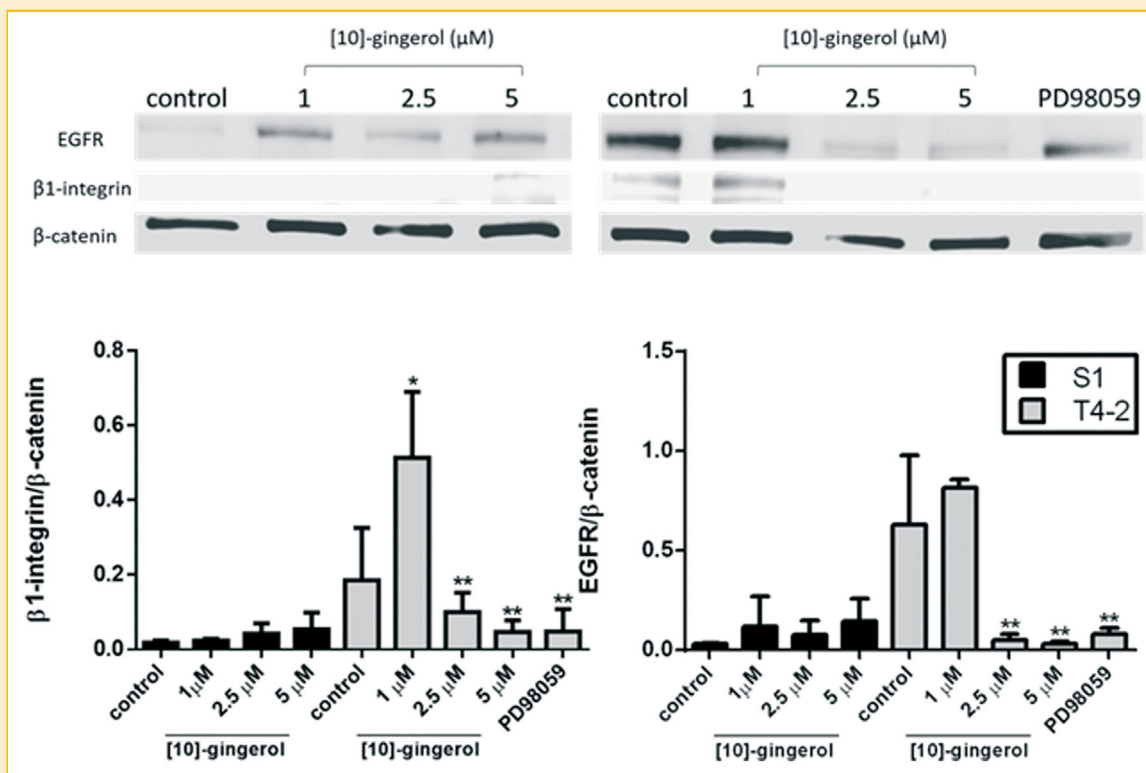


Fig. 2. [10]-gingerol reverts malignant phenotype in T4-2 cells by EGFR and β 1-integrin suppression. After treatments, the protein content from the supernatant ECM gels of 3D cell culture was measured, applied onto a SDS-PAGE gel and transferred to nitrocellulose membranes. Membranes were incubated with anti- β 1-integrin, -EGFR, and - β -catenin antibodies, followed by incubation with a HRP-conjugated secondary antibody. Beta-catenin was used as the endogenous control. The results showed that [10]-gingerol induces a downregulation of EGFR and β 1-integrin on T4-2 cells. Levels of EGFR and β 1-integrin in S1 cells levels were not significantly changed when treated with [10]-gingerol. The suppression is comparable to the mechanistic inhibitor of MAPK, PD98059 used as the control and occurs in a concentration-dependent fashion. * $P < 0.01$; ** $P < 0.001$.

cells [Weaver et al., 1997; Becker-Weimann et al., 2013; Onodera et al., 2014] in a process called “phenotypic reversion.” The resulting 3D morphology of the colonies is used to phenotypically distinguish malignant from non-malignant cell lines [Wang et al., 2002; Kenny et al., 2007].

In this study, we observed that [10]-gingerol, a natural product extracted from ginger, is able to revert the malignant phenotype in physiologically relevant 3D cultures of cancer cells growing in Ir-ECM. These effects are mediated by EGFR and β 1-integrin suppression. The results presented in this study are in agreement with previous studies demonstrating that [10]-gingerol presented anti-tumor activities in MDA-MB-231 breast cancer cells, which are mediated by EGFR suppression and inactivation of Akt and MAPK activities [Joo et al., 2016].

In our study, [10]-gingerol demonstrated to have little or null effects on non-malignant S1 cells. We have already shown that [10]-gingerol was able to inhibit cancer cell proliferation with more specificity compared to non-cancer cells [Almada da Silva et al., 2012]. In addition, in this study, we observed a high rate of luminal apoptosis on 3D structures when malignant cells were incubated with [10]-gingerol, with no effect on non-malignant cells. Few studies have demonstrated the effects of [10]-gingerol on apoptosis induction. Ryu and Chung [2015] showed that [10]-gingerol induces

mitochondrial apoptosis through activation of MAPK pathway in HCT116 human colon cancer cells.

Nevertheless, the apoptotic activity of [6]-gingerol was extensively demonstrated [Poltronieri et al., 2014]. The molecular mechanisms by which [6]-gingerol mediates apoptosis induction are diverse and include suppression of cyclin D1 expression by modulation of β -catenin, PKC ϵ , and GSK-3 β pathways [Lee et al., 2008], inhibition of NF- κ B, AP-1, COX-2, MAPK, pJNK, and pERK [Park et al., 2006], increase in p53 content [Lin et al., 2012], and caspase activation [Ishiguro et al., 2007; Shukla et al., 2007; Nigam et al., 2009; Chakraborty et al., 2012].

There are few reports showing the effects of natural products on the reversion of the malignant in 3D culture. One of these examples is resveratrol, which has been demonstrated to induce luminal apoptosis in 3D, but not in 2D culture leading to the formation of a luminal cavity, probably by inhibiting phosphodiesterase 4 (PDE4) activity [Tsunoda et al., 2014]. Whether [10]-gingerol is able to inhibit PDE4 activity remains to be investigated.

Studies using monolayer cultures are not able to mimic a physiological relevant microenvironment found in vivo, which is well-known to interfere massively in signaling pathways. In fact, the capability of [10]-gingerol to reduce the expression of EGFR and β 1-integrin, as well as its apoptotic activity, could be correlated to some of

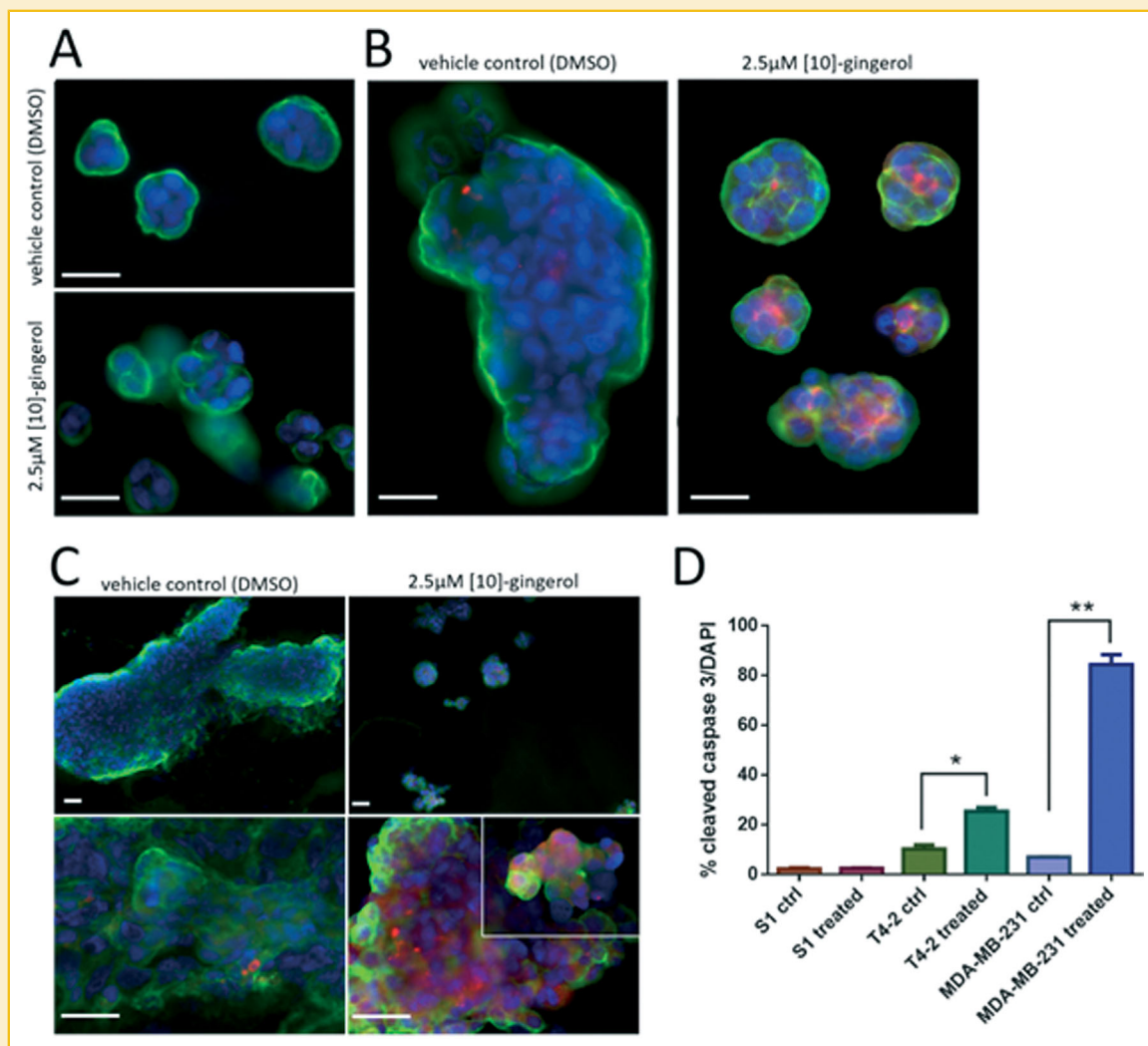


Fig. 3. Cleaved caspase 3 immunostaining of (A) S1 vehicle-treated cells (upper panel) and S1 [10]-gingerol-treated cells (lower panel); (B) T4-2 vehicle-treated cells and T4-2 [10]-gingerol-treated cells; (C) MDA-MB-231 [10]-gingerol treated cells. The 3D structures were collected from the wells in which they were grown, fixed with 4% paraformaldehyde and stained with anti- β 1-integrin (A1B2 antibody, green), anti- β -catenin (red), and with the Alexa Fluor secondary antibody, for immunostaining procedures. Nuclei were stained with DAPI (blue). S1 cells treated with vehicle and 2.5 μ M [10]-gingerol are negative to cleaved caspase 3. T4-2 treated reverted structures have luminal localized apoptosis. Treated MDA-MB-231 colonies are extensive positive to cleaved caspase 3. (D) Graph of fluorescence mean intensity, showing the increase in cleaved-caspase 3 in [10]-gingerol treated T4-2 and MDA-MB-231 colonies. Magnification is described in the figures. Scale bars 40 μ m. * P < 0.01; ** P < 0.001.

the signaling pathways cited above, however, more studies on 3D models and in vivo are necessary to reveal its role in these cellular events.

CONCLUSION

In summary, the results obtained showed that [10]-gingerol changed the morphology of malignant cell structures in Ir-ECM 3D cell culture, without interfering in non-malignant S1 cells, showing selectivity of [10]-gingerol against malignant cell lines. The compound was also able to revert the malignant phenotype of T4-2 cells by downregulation of EGFR and β 1-integrin expression and induction of apoptosis. These results indicate that [10]-gingerol may be an effective compound to be used as adjuvant therapy in breast cancer treatment.

ACKNOWLEDGMENTS

This research was supported by the São Paulo Research Foundation (FAPESP, grants 2012/18908-6, 2013/00798-2, and 2015/08146-0). We would like to thank Dr. Jamie Acosta, who provided insight and expertise that greatly assisted the research and for his critical review of this manuscript.

REFERENCES

Almada da Silva J, Becceneri AB, Sanches Mutti H, Moreno Martin AC, Fernandes da Silva MF, Fernandes JB, Vieira PC, Cominetti MR. 2012. Purification and differential biological effects of ginger-derived substances on normal and tumor cell lines. *J Chromatogr B Analyt Technol Biomed Life Sci* 903:157–162.

- Becker-Weimann S, Xiong G, Furuta S, Han J, Kuhn I, Akavia UD, Pe'er D, Bissell MJ, Xu R. 2013. NF κ B disrupts tissue polarity in 3D by preventing integration of microenvironmental signals. *Oncotarget* 4:2010–2020.
- Bissell MJ, Rizki A, Mian IS. 2003. Tissue architecture: The ultimate regulator of breast epithelial function. *Curr Opin Cell Biol* 15:753–762.
- Briand P, Petersen OW, Van Deurs B. 1987. A new diploid nontumorigenic human breast epithelial cell line isolated and propagated in chemically defined medium. *In Vitro Cell Dev Biol* 23:181–188.
- Chakraborty D, Bishayee K, Ghosh S, Biswas R, Mandal SK, Khuda-Bukhsh AR. 2012. [6]-Gingerol induces caspase 3 dependent apoptosis and autophagy in cancer cells: Drug-DNA interaction and expression of certain signal genes in HeLa cells. *Eur J Pharmacol* 694:20–29.
- Haniadka R, Rajeev AG, Palatty PL, Arora R, Baliga MS. 2012. *Zingiber officinale* (ginger) as an anti-emetic in cancer chemotherapy: A review. *J Altern Complement Med* 18:440–444.
- Ishiguro K, Ando T, Maeda O, Ohmiya N, Niwa Y, Kadomatsu K, Goto H. 2007. Ginger ingredients reduce viability of gastric cancer cells via distinct mechanisms. *Biochem Biophys Res Commun* 362:218–223.
- Joo JH, Hong SS, Cho YR, Seo DW. 2016. 10-Gingerol inhibits proliferation and invasion of MDA-MB-231 breast cancer cells through suppression of Akt and p38MAPK activity. *Oncol Rep* 35:779–784.
- Kenny PA, Lee GY, Myers CA, Neve RM, Semeiks JR, Spellman PT, Lorenz K, Lee EH, Barcellos-Hoff MH, Petersen OW, Gray JW, Bissell MJ. 2007. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Mol Oncol* 1:84–96.
- Lee GY, Kenny PA, Lee EH, Bissell MJ. 2007. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat Methods* 4:359–365.
- Lee SH, Cekanova M, Baek SJ. 2008. Multiple mechanisms are involved in 6-gingerol-induced cell growth arrest and apoptosis in human colorectal cancer cells. *Mol Carcinog* 47:197–208.
- Lin CB, Lin CC, Tsay GJ. 2012. 6-Gingerol inhibits growth of colon cancer cell LoVo via induction of G2/M arrest. *Evid Based Complement Alternat Med* 2012:326096.
- Liu Y, Whelan RJ, Pattnaik BR, Ludwig K, Subudhi E, Rowland H, Claussen N, Zucker N, Uppal S, Kushner DM, Felder M, Patankar MS, Kapur A. 2012. Terpenoids from *Zingiber officinale* (ginger) induce apoptosis in endometrial cancer cells through the activation of p53. *PLoS ONE* 7:1–10.
- Nigam N, Bhui K, Prasad S, George J, Shukla Y. 2009. [6]-Gingerol induces reactive oxygen species regulated mitochondrial cell death pathway in human epidermoid carcinoma A431 cells. *Chem Biol Interact* 181:77–84.
- Onodera Y, Nam JM, Bissell MJ. 2014. Increased sugar uptake promotes oncogenesis via EPAC/RAP1 and O-GlcNAc pathways. *J Clin Invest* 124:367–384.
- Park YJ, Wen J, Bang S, Park SW, Song SY. 2006. [6]-Gingerol induces cell cycle arrest and cell death of mutant p53-expressing pancreatic cancer cells. *Yonsei Med J* 47:688–697.
- Petersen OW, Ronnov-Jessen L, Howlett AR, Bissell MJ. 1992. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proc Natl Acad Sci USA* 89:9064–9068.
- Poltronieri J, Becceneri AB, Fuzer AM, Filho JC, Martin AC, Vieira PC, Pouliot N, Cominetti MR. 2014. [6]-Gingerol as a cancer chemopreventive agent: A review of its activity on different steps of the metastatic process. *Mini Rev Med Chem* 14:313–321.
- Rizki A, Weaver VM, Lee SY, Rozenberg GI, Chin K, Myers CA, Bascom JL, Mott JD, Semeiks JR, Grate LR, Mian IS, Borowsky AD, Jensen RA, Idowu MO, Chen F, Chen DJ, Petersen OW, Gray JW, Bissell MJ. 2008. A human breast cell model of preinvasive to invasive transition. *Cancer Res* 68:1378–1387.
- Ryu MJ, Chung HS. 2015. [10]-Gingerol induces mitochondrial apoptosis through activation of MAPK pathway in HCT116 human colon cancer cells. *In Vitro Cell Dev Biol Anim* 51:92–101.
- Schmeichel KL, Weaver VM, Bissell MJ. 1998. Structural cues from the tissue microenvironment are essential determinants of the human mammary epithelial cell phenotype. *J Mammary Gland Biol Neoplasia* 3:201–213.
- Shukla Y, Prasad S, Tripathi C, Singh M, George J, Kalra N. 2007. In vitro and in vivo modulation of testosterone mediated alterations in apoptosis related proteins by [6]-gingerol. *Mol Nutr Food Res* 51:1492–1502.
- Thoma CR, Zimmermann M, Agarkova I, Kelm JM, Krek W. 2014. 3D cell culture systems modeling tumor growth determinants in cancer target discovery. *Adv Drug Deliv Rev* 69–70:29–41.
- Tsunoda T, Ishikura S, Doi K, Matsuzaki H, Iwaihara Y, Shirasawa S. 2014. Resveratrol induces luminal apoptosis of human colorectal cancer HCT116 cells in three-dimensional culture. *Anticancer Res* 34:4551–4555.
- Vidi PA, Bissell MJ, Lelievre SA. 2013. Three-dimensional culture of human breast epithelial cells: The how and the why. *Methods Mol Biol* 945:193–219.
- Wang F, Hansen RK, Radisky D, Yoneda T, Barcellos-Hoff MH, Petersen OW, Turley EA, Bissell MJ. 2002. Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts. *J Natl Cancer Inst* 94:1494–1503.
- Wang F, Weaver VM, Petersen OW, Larabell CA, Dedhar S, Briand P, Lupu R, Bissell MJ. 1998. Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: A different perspective in epithelial biology. *Proc Natl Acad Sci USA* 95:14821–14826.
- Weaver VM, Bissell MJ. 1999a. Functional culture models to study mechanisms governing apoptosis in normal and malignant mammary epithelial cells. *J Mammary Gland Biol Neoplasia* 4:193–201.
- Weaver VM, Bissell MJ. 1999b. Functional culture models to study mechanisms governing apoptosis in normal and malignant mammary epithelial cells. *J Mammary Gland Biol Neoplasia* 4(2):193–201.
- Weaver VM, Fischer AH, Peterson OW, Bissell MJ. 1996. The importance of the microenvironment in breast cancer progression: Recapitulation of mammary tumorigenesis using a unique human mammary epithelial cell model and a three-dimensional culture assay. *Biochem Cell Biol* 74(6):833–851.
- Weaver VM, Lelièvre S, Lakins JN, Chrenek MA, Jones JCR, Giancotti F, Werb Z, Bissell MJ. 2002. β 4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. *Cancer Cell* 2(3):205–216.
- Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, Bissell MJ. 1997. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *J Cell Biol* 137:231–245.
- Weigelt B, Ghajar CM, Bissell MJ. 2014. The need for complex 3D culture models to unravel novel pathways and identify accurate biomarkers in breast cancer. *Adv Drug Deliv Rev* 69–70:42–51.