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Full Length Research Paper

Rhodiola crenulata inhibits the tumorigenic properties of invasive mammary epithelial cells with stem cell characteristics

Kelly J.Gauger[1,2], Adaris Rodríguez-Cortés[2], Malgorzata Hartwich[4] and Sallie Smith Schneider[1,3*]

[1]Pioneer Valley Life Sciences Institute, Baystate Medical Center, Springfield, MA 01199, USA.
[2]Biology Department, University of Massachusetts, Amherst, MA 01003, USA.
[3]Veterinary and Animal Sciences, University of Massachusetts, Amherst, MA 01003, USA.
[4]Am Vollmersbusch 23, D-58706 Menden, Germany.

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***Rhodiola* is a high altitude perennial plant that is widespread in Eastern Europe and Asia. The root of the *Rhodiola* plant has been used for many years in eastern traditional medicine and it is well known for cardio-protective and central nervous system enhancement effects. It is considered a plant-derived "adaptogen" which is capable of maintaining a physiological homeostasis upon exposure to stress. The *Rhodiola rosea* and *Rhodiola crenulata* species have been shown to elicit anti-tumor activities *in vitro* and *in vivo*. Here we show that *R. crenulata* is able to block several characteristics associated with cancer stem cell behavior including migration, invasion, resistance to anoikis and tumorsphere formation. In addition, we show that *R. crenulata* regulates the mRNA expression levels of Id (Inhibitor of differentiation or Inhibitor of DNA-binding) proteins, which play several key roles in development, cell cycle regulation and tumorigenesis.**

Key words: Breast cancer, *rhodiola*, metastasis, stem cells, $CD44^{high}/CD24^{low}$, anoikis, SFRP1, Id-1, Id-2, Id-3.

INTRODUCTION

An assortment of chemical compounds is currently being investigated in preclinical analyses for their ability to act as both chemopreventive and chemotherapeutic agents. In particular, there is a growing enthusiasm for the use for botanical medicines in the battle against cancer. *Rhodiola* is an adaptogenic genus of plants that exerts a variety health promoting effects, including anti-tumor effects.

There are approximately 90 different species of *Rhodiola* that grow in Europe and Asia and a handful of these plants have been used in traditional eastern medicine. Typically, these plants are found growing at high altitudes in barren soils and it is believed that the adaptation to grow under these conditions contributes to some of the protective and medicinal activities of *Rhodiola*. The *Rhodiola rosea* species has been shown to have anti-cancer effects in both animals and humans (Dement'eva and Iaremenko, 1987; Udintsev and Shakhov, 1989; Udintsev and Shakhov, 1991; Majewska et al., 2006; Bocharova et al., 1995; Kormosh et al., 2006; Razina et al., 2000; Skopinska-Rozewska et al., 2008; Skopinska-Rozewska et al., 2008; Udintsev et al., 1992; Zhang et al., 2007). Recently, it has been shown that *Rhodiola crenulata* also has anti-cancer effects (Tu et al., 2008). Tu et al found that *R. crenulata* renders both mouse and human breast cancer cells more susceptible to cell death and it significantly inhibits the migratory and invasive behaviors exhibited by breast cancer cells.

Furthermore, *R. crenulata* fed to mice injected with breast cancer cells increases the survival time of tumor graft-bearing mice and also prevents tumor establishment (Tu et al., 2008). Breast cancers progress through stages of increasing malignancy by genetic and epigenetic mutations that promote their growth, invasiveness and metastasis. Aberrant activation of the Wnt/β-catenin

*Corresponding author. E-mail: sallie.schneider@baystatehealth.org.

pathway contributes to the genesis of a wide range of human cancers, including breast cancer, by promoting malignant behavior. Secreted frizzled-related proteins (SFRPs) are a family of proteins that antagonize Wnt signaling and it has been shown that SFRP1 is lost in breast cancer cell lines as well as human breast tumor specimens (Klopocki et al., 2004; Wong et al., 2002; Zhou et al., 1998). We recently reported that knock down of SFRP1 in a non-malignant epithelial cell line, 76N TERT (TERT-siSFRP1 cells), results in cells that acquire tumorigenic properties that are characteristic of aggressive breast cancer cells. These characteristics include resistance to anoikis (anchorage-independent cell death), up-regulation of CD44 and down-regulation of CD24 cell surface markers and increased invasive behaviors (Gauger et al., 2009).

It has been recently accepted that mammary stem cells reside in adult breast tissue and give rise to progenitor cells that generate differentiated progeny (Russo et al., 2006). The presence of stem cells in the mouse mammary gland has been clearly demonstrated by several researchers who show that a single stem cell can repopulate a cleared fat pad (Shackleton et al., 2006; Stingl et al., 2006; Liao et al., 2007). It has been shown that mammary stem cells are enriched with a population of cells that can grow under anchorage-independent (AIG) conditions. When in non-adherent culture conditions these cells are capable of surviving, proliferating and forming discrete clusters of cells termed “mammospheres”. Trypsinization and replating of the mammospheres results in secondary and tertiary mammospheres, indicating the maintenance and the renewal of the stem cells, whereas non-stem cells lose their ability to form spheres by the third round of replating. The mammospheres are enriched in progenitor cells capable of differentiating along multiple mammary epithelial cell lineages (Dontu et al., 2003). Mammary carcinomas also have a population of cells that are stem-like in that they can form “tumorspheres” when in non-adherent conditions. Very few cells are required to form tumors and they possess similar behaviors and cell surface markers as those of normal stem cells (Cariati et al., 2008; Ponti et al., 2005). It has been proposed that one of the mechanisms by which tumors arise is either via mutations that impart cancer properties to a stem or progenitor cell that would have otherwise had a defined life span, or through the de-differentiation of a mutated epithelial cell into a stem/progenitor-like cell. Al-Hajj et al., 2003 have found that as few as 100 cells with a distinct stem cell surface marker expression pattern of $CD44^{high}/CD24^{low}$ can induce mammary tumor formation in mice (Al-Hajj et al., 2003).

Considering that breast cancer stem cells are remarkably resistant to traditional chemotherapeutic regimens, the work described hereafter was undertaken to determine if *R. crenulata* is capable of attenuating some of the malignant characteristics associated with human breast cancer cells with a stem-like phenotype. Both TERT-siSFRP1 cells and human cancer invasive MDA-MB-231 cells exhibit several of the stem cell-like properties previously described (Gauger et al., 2009; Sheridan et al., 2006). We clearly demonstrate that *R. crenulata* blocks invasion as well as migration, promotes anoikis and prevents tumorsphere formation in TERT-siSFRP1 and MDA-MB-231 cells. Moreover, *R. crenulata* alters the expression of the Id family of genes, which are known to regulate these very processes.

MATERIALS AND METHODS

R. crenulata preparation

R. crenulata root extract that was optimized for phenolic content was obtained from Barrington Nutritionals (Harrison, NY). The powder form of the extract was dissolved in 10% ethanol and filter-sterilized distilled water.

Cell culture

All cell lines were routinely cultivated at 37°C under 5% CO_2. TERT-siSFRP1 cells were maintained in DMEM/F12 (GIBCO, Grand Island, NY) supplemented with the following components, from GIBCO: 1% FBS, 1X Antibiotic - Antimycotic (100X) and 20 µg/ml Gentamycin and 2 µg/ml puromycin and from Sigma (St Louis, MO): 50 µM L(+)-Ascorbic acid sodium salt, 1 ng/ml Cholera Toxin Vibrio, 12.5 ng/ml Epidermal Growth Factor murine submaxillary, 2 nM Estradiol, 0.1 mM Ethanolamine, 1 µg/ml Hydrocortisone-Water Soluble, 1 µg/ml human Insulin solution, 0.1 mM O-Phosphorylethanolamine, 35 µg/ml bovine pituitary extract, 15 nM Sodium selenite, 10 µg/ml human apo-Transferrin and 10 nM 3,3',5-Triiodo-L-thyronine sodium salt. MDA-MB-231 cells were maintained in DMEM supplemented with 10% FBS and 20 µg/ml Gentamycin (GIBCO).

Migration and invasion assay

For the scratch-wound assay, TERT-siSFRP1 cells were plated in 30 mm dishes and allowed to reach 100% confluence. A pipette tip was utilized to generate a wound (scratch) down the center of the plate. Images of the cells that migrated across the scratch wound were captured with Nikon Eclipse TE2000-U using Metaview™ software (Universal Imaging Corporation).

For chamber assays, TERT-siSFRP1 cells were grown to 70% confluence and pre-treated with either 0.1% EtOH or 100 µg/ml *R. crenulata*. After 24 h, the pre-treated cells were trypsinized, centrifuged at 1,000 g for 3 min and brought to a concentration of 1×10^6 cells/ml in serum-free media. 5×10^5 cells/well were seeded in serum free media either in BD BioCoat control chambers or in Matrigel invasion chambers (BD Biosciences) above media containing 10% FBS. Following 22 h incubation, chambers were removed and cells were fixed for 10 min in 10% formalin, stained for 10 min with 10% Crystal Violet and rinsed 3 times with dH_20. Non-migrating/invading cells were removed from the upper surface of the membrane by scrubbing the insert with cotton tipped swab moistened with 1× PBS. The insert was then removed from the chamber with a scalpel, and mounted on a microscope slide in Cytoseal™XYL mounting medium (Richard-Allan Scientific). Images were captured with an Olympic BX41 light microscope using SPOTSOFTWARE (Diagnostic Instruments, Inc, Sterling Heights, MI).

Fluorescence activated cell sorting *(FACS)*

TERT-siSFRP1 cells were grown to 70% confluence and pre-treated with either 0.1% EtOH or 100 µg/ml *R. crenulata*. MDA-MB-231 cells were treated in the same way, but were grown in serum-free media. 30 mm dishes were coated with 2 ml of 1% agarose/DMEM, which was allowed to polymerize creating a barrier that would prevent cellular attachment. 24 h post-treatment, cells were trypsinized, plated in the 1% agarose/DMEM coated dishes and treated with 0.1% EtOH or 100 µg/ml *R. crenulata*. After an additional 24 or 48 h, the cell-containing media was collected and cells were pelleted by centrifugation (3 min, 1000 × g). The pellet was re-suspended in ice-cold 1X PBS, transferred into a round bottom 12 × 75 mm plastic culture tube (VWR international, NJ, USA) and incubated with 1 µg/ml propidium iodine (Invitrogen) in the dark for 15 min on ice to stain dead cells. The ratio of propidium iodine positive/total cells was determined by flow cytometry (BD FACS Calibur, BD Bioscience and San Jose, CA).

Tumorsphere formation assay

TERT-siSFRP1 and MDA-MB-231 cells were trypsinized and the cell pellet was collected by centrifugation (3 min, 1000 × g). Cells were suspended again in growth media and carefully pipetted to dissociate the cells into a single cell suspension. A total of 500 cells were plated in 96-well Ultra-Low Attachment plates (CORNING, Lowell, MA) and wells were examined to ensure that there were only single cells in the wells. These cells were treated with 0.1% EtOH or 100 µg/ml *R. crenulata* every other day for 10 days. Images of tumorsphere formation were captured with Nikon Eclipse TE2000-U using Metaview software and on day 10, the total number of tumorspheres that formed was counted.

RNA Isolation and Real-Time PCR analyses

TERT-siSFRP1 and MDA-MB-231 cells were treated with 0.1% EtOH (vehicle) or 100 µg/ml *R. crenulata* and 0.1% EtOH and after 24 or 48 h, total RNA was extracted from cells using an acid-phenol extraction procedure (Chomczynski and Sacchi, 1987) according to the manufacturer's instructions (Trizol, Invitrogen, Carlsbad, CA). Relative levels of mRNA were determined by quantitative real-time Polymerase Chain Reaction (PCR) using the Mx4000 real time PCR system (Stratagene, La Jolla, CA). All values were normalized to the amplification of Actin mRNA. The PCR primer sequences used are as follows: Id-1 forward; 5'-CCAGAACCGCAAGGTGAG-3', Id-1 reverse; 5'-GGTCCCTGATGTAGTCGATGA-3', Id-2 forward; 5'-GACAGAACCAGGCGTCCA-3', Id-2 reverse; 5'-AGCTCAGAAGGGAATTCAGATG-3', Id-3 forward; 5'-CATCTCCAACGACAAAAGGAG-3'; Id-3 reverse; 5'-CTTCCGGCAGGAGGGTT-3'; Actin forward; 5'-CCAACCGCGAGAAGATGA-3'; Actin reverse; 5'-CCAGAGGCGTACAGGGATAG-3'. The assays were performed using the 1-Step 2X Brilliant SYBR Green QRT-PCR Master Mix Kit (Stratagene) containing 200 nM forward primer, 200 nM reverse primer and 100 ng of total template RNA. The conditions for cDNA synthesis and target mRNA amplification were performed as follows: 1 cycle of 50°C for 30 min; 1 cycle of 95°C for 10 min; and 35 cycles each of 95°C for 30 s, 55°C for 1 min and 72°C for 30 s.

Statistical analyses

All results were analyzed using a student's t-test (Prizm, GraphPad Software, Inc, San Diego, CA).

RESULTS AND DISCUSSION

Cellular metastasis takes place when epithelial cells in a primary tumor leave their site of origin, digest and break through the extracellular matrix, migrate into blood vessels and invade secondary sites. We have previously shown that TERT-siSFRP1 cells are an extremely migratory and invasive mammary epithelial cell line (Gauger et al., 2009).

Since *R. crenulata* inhibits the migration and invasion of MDA-MB-231 breast cancer cells (Tu et al., 2008), we hypothesized that the motile and invasive behavior observed in TERT-siSFRP1 cells would be abolished if they were grown in the presence of *R. crenulata*. Considering that metastasis is likely to occur when cells become more migratory, we decided to test the effects of *R. crenulata* on TERT-siSFRP1 cells utilizing a simple scratch wound assay (Figure 1a). The images that we obtained illustrated that after just 3 h, TERT-siSFRP1 cells treated with 0.1% EtOH (vehicle) began to migrate and fill in the wound while treatment of cells with 100 µg/ml *R. crenulata* prevented the motility of these cells. After 6 h, *R. crenulata* continued to block migration. At 24 h, TERT-siSFRP1 cells completely filled in the scratch wound whereas when cells were grown in the presence of *R. crenulata*; at 24 h wound closure had just begun. Next, cells were plated in BD BioCoat control chambers or Matrigel Invasion Chambers (BD Biosciences). The cells capable of migrating through the 8 µm pore towards a chemoattractant were stained with 10% crystal violet (Figure 1b). Quantification of the stained cells revealed that TERT-siSFRP1 cells treated with *R. crenulata* were significantly less migratory than vehicle treated cells (Figure 1c). Cells that were able to invade the reconstituted basement membrane (Matrigel) through an 8 m pore towards a chemoattractant were stained with 10% crystal violet. Figure 1B clearly illustrates and Figure 1C quantitatively confirms that *R. crenulata* blocked the invasiveness of TERT-siSFRP1 cells.

The pathogenesis of breast cancer involves the aberrant expression and altered function of the genes involved in cell-cycle regulation and cell death. Tu et al previously demonstrated that *R. crenulata* induces cell death in both human and murine breast cancer cell lines in the absence of serum (Tu et al., 2008). Therefore, we sought to determine whether *R. crenulata* would cause cell death in TERT-siSFRP1 cells. We found that *R. crenulata* treated TERT-siSFRP1 cells underwent signifi-cantly more death both in the presence and absence of irradiation (IR), although IR treatment did not further enhance *R. crenulata* induced cell death (data not shown). Normal non-malignant adherent cells are strongly dependent on adhesion to the extracellular matrix for cell proliferation and undergo cell death if they are detached from the substratum, in a process known as anoikis (Frisch and Francis, 1994) and resistance to anoikis has been suggested as a prerequisite for cancer

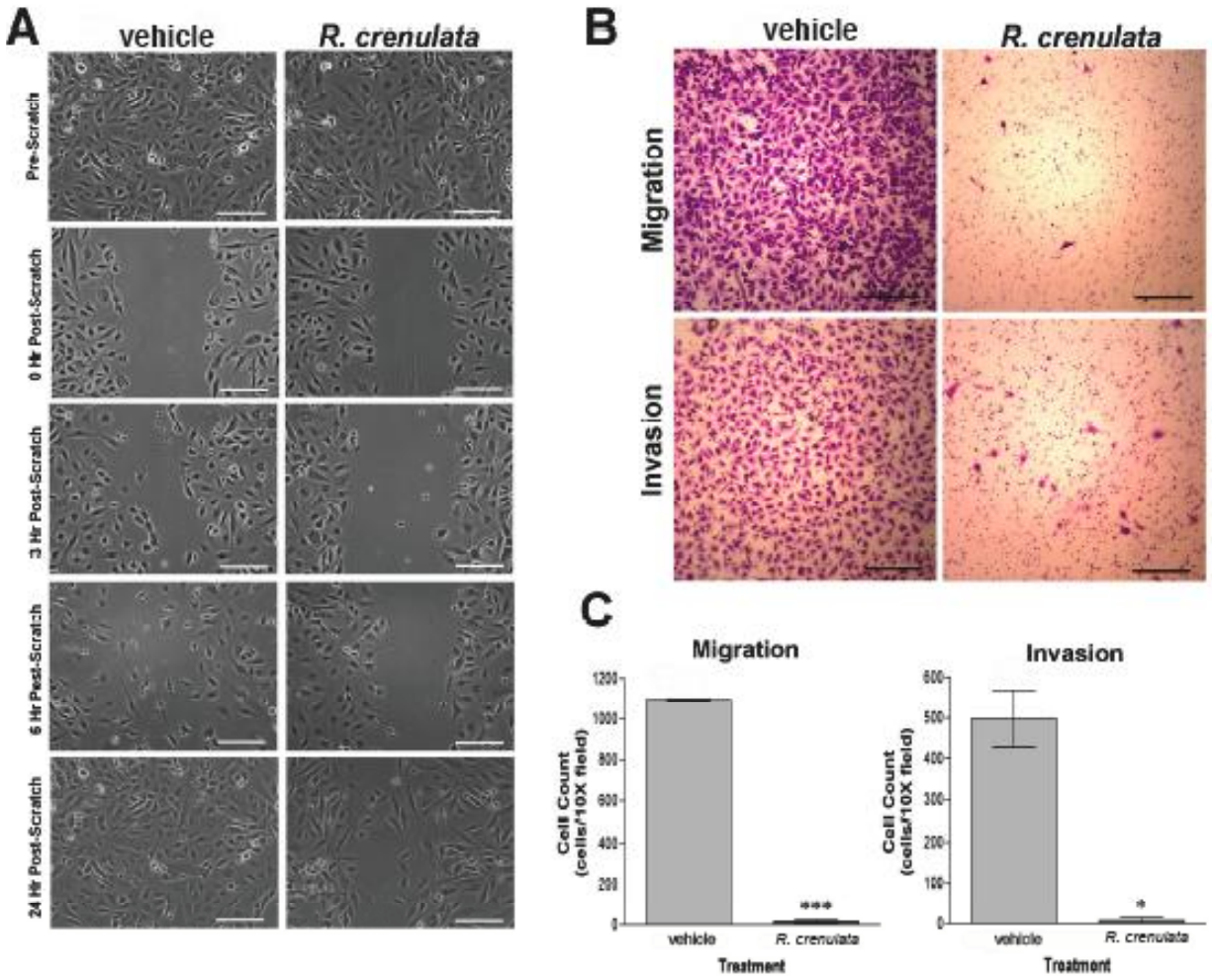

Figure 1. *R. crenulata* blocks the cellular migration and invasion of TERT-siSFRP1 cells. (A) TERT-siSFRP1 cells were plated in 30 mm dishes and allowed to reach 100% confluence and treated with vehicle or *R. crenulata*. A pipette tip was utilized to generate a wound (scratch) down the center of the plate. Images illustrate cells capable of migrating across the scratch 3, 6, and 24 hours after the wound was created. Images were captured at 20X magnification; scale bar 100 µm. (B) TERT-siSFRP1 cells treated with vehicle or *R. crenulata* were plated in BD BioCoat control inserts or Matrigel invasion chambers. Cells capable of migrating through the 8 m pore or invading the reconstituted basement membrane (Matrigel) through the 8 m pore towards a chemoattractant were stained with 10% crystal violet and counted. Images were captured at 10X magnification; scale bar 500 µm. (C) Experiments were repeated three times and the numbers of cells within a representative 10X field from each experiment were counted. The bars represent SEM of the cell number. *p<0.05,***p<0.001 (significantly different from vehicle treated cells using a student's *t*-test).

cells to metastasize. In contrast, tumor cells can survive and grow without adhesion to a substratum. With this in mind we next tested the ability of *R. crenulata* to sensitize the anoikis-resistant TERT-siSFRP1 cells to anchorage-independent cell death. Twenty-four hours after seeding the cells, FACs analysis of Propidium Iodide incorporation was performed to compare events of cell death between vehicle and *R. crenulata* treated cells. We found that significantly more cell death occurred when cells were grown unattached and in the presence of *R. crenulata* (Figure 2a). Moreover, after 48 h of *R. crenulata* treatment, TERT-siSFRP1 cells remained susceptible to anchorage-independent induced cell death (Figure 2a). Next, we sought to determine whether an established invasive breast cancer cell line, MDA-MB-231 cells, would be sensitized to anoikis when grown in the presence of *R. crenulata*. We clearly show that, in anchorage-independent growth conditions at 24 and 48 h, significantly more *R. crenulata* treated MDA-MB-231 cells died as compared to vehicle treated control cells (Figure 2b).

Both non-malignant and malignant mammary epithelial cells with stem-cell characteristics, including a $CD44^{high}/CD24^{low}$ cell surface marker pattern, are capable of forming mammosphere/tumorspheres in suspension culture (Ponti et al., 2005). It has previously been

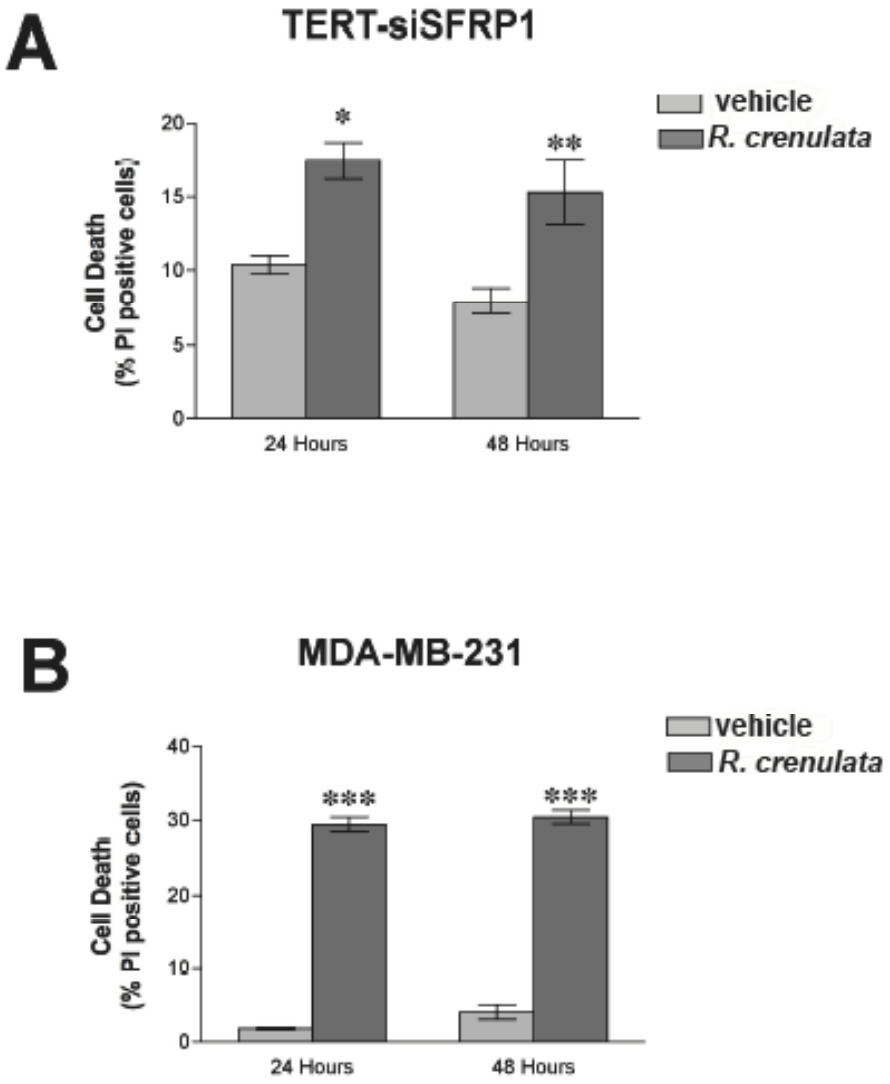

Figure 2. *R. crenulata* induces anoikis in TERT-siSFRP1 and MDA-MB-231 cells. Cell culture dishes were coated with 1% agarose/DMEM, which was allowed to polymerize and create a barrier that prevented cellular attachment. (A) TERT-siSFRP1 cells and (B) MDA-MB-231 cells were plated in the coated wells, treated with vehicle or *R. crenulata*. After 24 and 48 h, FACs analysis was performed to compare the percentage of propidium iodide positive (PI) (FL2; dead) to PI negative (live) cells within each cell line. Two separate experiments were performed in triplicate. Bars represent SEM of the relative cell death (FL2 positive cells/total cells). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (significantly different from vehicle treated cells using student's *t*-test).

demonstrated that both TERT-siSFRP1 cells and MDA-MB-231 cells exhibit the $CD44^{high}/CD24^{low}$ phenotype and form tumorspheres when cultured under the appropriate conditions (Gauger et al., 2009; Sheridan et al., 2006). We first wanted to determine whether *R. crenulata* affects the $CD44^{high}/CD24^{low}$ phenotype exhibited by these cell lines and found that there was no difference between vehicle and *R. crenulata* treated cells (data not shown). Next, to establish whether *R. crenulata* is able to abolish mammosphere formation, we seeded TERT-siSFRP1 and MDA-MB-231 cells at a single cell density under conditions that would allow for tumorsphere formation and treated with vehicle or *R. crenulata* for 10 days. Figure 3a illustrates that when cells were treated with *R. crenulata*, the cells were unable to form tumorspheres. We then quantified the extent to which *R. crenulata* impeded the ability of TERT-siSFRP1 and MDA-MB-231 cells from forming the spherical structures by counting the total number of clusters. Our data clearly indicate that there was a significant reduction in tumorsphere development (Figure 3b).

Epithelial to mesenchymal transitions (EMT) are

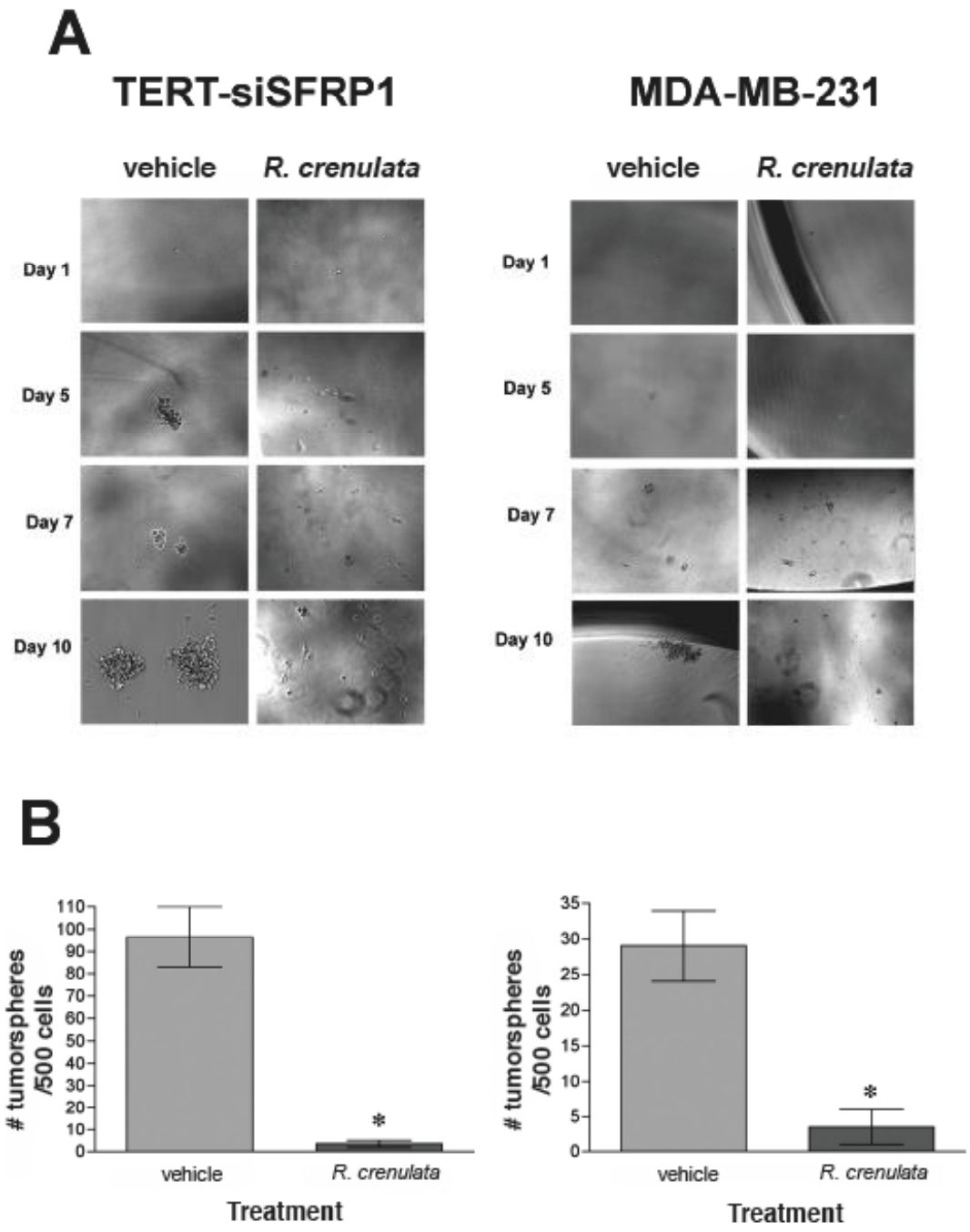

Figure 3. *R. crenulata* blocks tumorsphere formation. A total of 500 TERT-siSFRP1 and 500 MDA-MB-231 cells were plated in low-attachment 96 well plates, treated with vehicle or *R. crenulata,* and cultured for 10 days. (A) Phase contrast images were captured at days 1, 5, 7 and 10. Images were captured at 20X magnification; (B) *In vitro* quantification of tumorspheres formed by cells as described. Bars represent SEM of the number of spheres formed/500 seeded cells. *$p < 0.05$ (significantly different from vehicle treated cells using student's *t*-test).

implicated in the conversion of early stage tumors into invasive malignancies. EMT is a process where epithelial cells lose polarity, lose cell-cell contacts and hence cell-cell contact inhibition, and undergo a dramatic remodeling of their cytoskeletons, resulting in cells with a mesenchymal morphology. Interestingly, Mani et al. 2008, showed that mammary epithelial cells forced to undergo EMT acquired stem cell-like characteristics (Mani et al., 2008). In addition to the stem-cell characteristics observed in TERT-siSFRP1 and MDA-MB-231 cells, these cell lines both possess a fibroblast-like morphology and express several genes associated with EMT in a

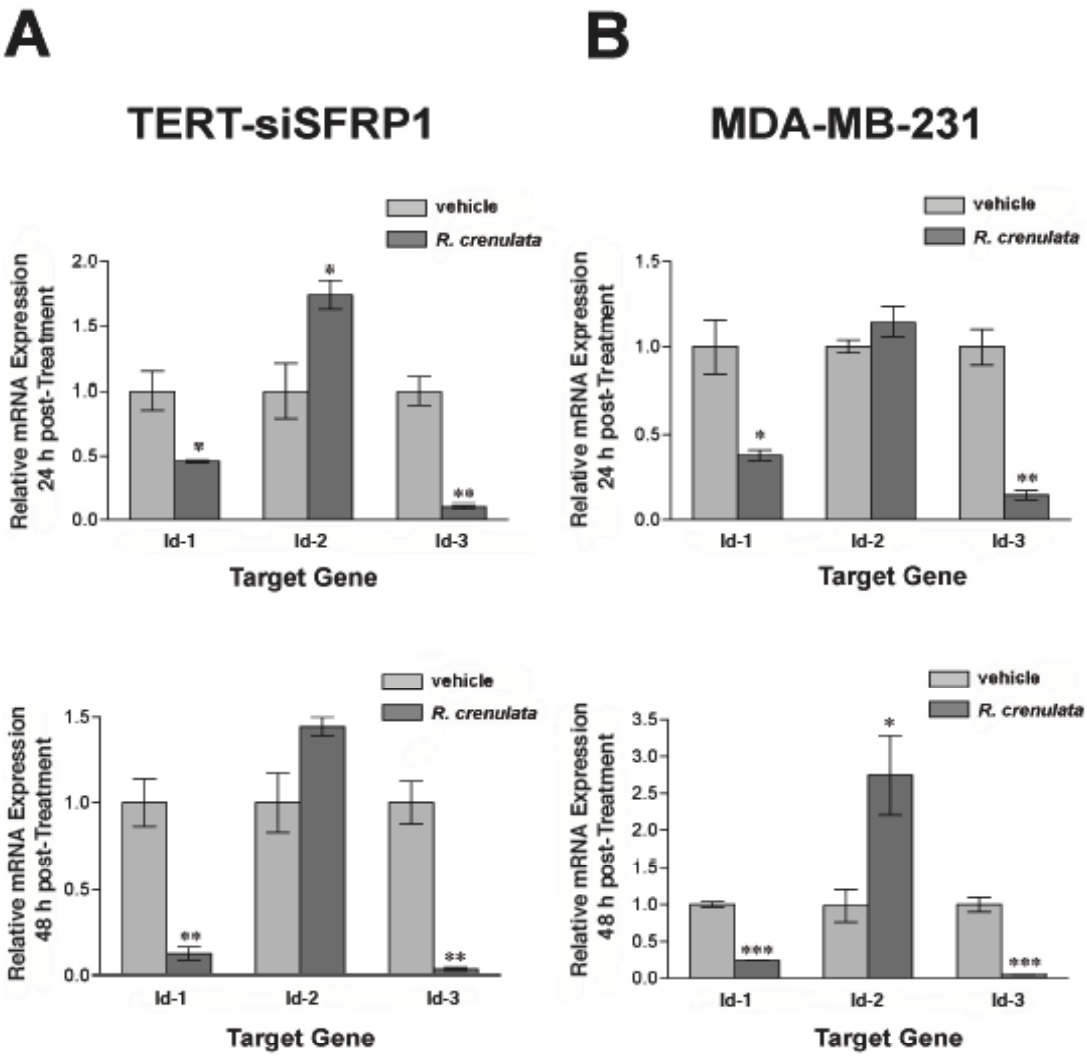

Figure 4. *R. crenulata* alters the expression of Id proteins in TERT-siSFRP1 and MDA-MB-231 cells. For real-time PCR analysis of Id-1, Id-2, and Id-3 gene expression, total RNA was isolated from three separate harvests of each cell line. Isolations were performed at either (A) 24 h or (B) 48 h after vehicle or *R. crenulata* treatments. Real-time PCR experiments were carried out twice. The results shown represent experiments performed in triplicate and normalized to the amplification of actin mRNA. Bars represent mean SEM of the fold change with respect to vehicle treated cells. *p<0.05, ***p<0.001, ***p<0.001 (significantly different from vehicle treated cells using student's *t*-test).

pattern that is consistent with the pathophysiological phenotype of EMT (Gauger et al., 2009; Olmeda et al., 2007; Mbalaviele et al., 1996; Bindels et al., 2006; Eger et al., 2005). Since the data described thus far demonstrates that *R. crenulata* impedes several behaviors associated with stemness and EMT, we wanted to establish whether the genes implicated in EMT were affected by *R. crenulata* treatment. Real-time PCR analysis of well characterized EMT associated genes revealed that the mRNA expression of these genes did not differ between vehicle and *R. crenulata* treated cells (data not shown).

The Id (Inhibitor of differentiation or inhibitor of DNA-binding) proteins are basic Helix-Loop-Helix (bHLH) proteins that lack a DNA-binding region and act as dominant negative inhibitors of other bHLH transcription factors that regulate differentiation-specific gene expression. The Id proteins play several critical roles in both normal and transformed tissues, including the mammary gland (Desprez et al., 2003). These proteins are able to promote cell proliferation and survival by activating growth promoting and blocking tumor suppressive pathways (Yokota and Mori, 2002; Ling et al., 2003; Swarbrick et al., 2005; Ling et al., 2002). Ids have also been implicated in stem cell renewal and maintenance and are often downregulated during stem cell

differentiation (Jung et al., 2009; Nogueira et al., 2000; Perry et al., 2007). It has been previously shown that Id-1 is frequently up-regulated in many types of human cancer and that its expression levels are also associated with an advanced tumor stage (Wong et al., 2004). In addition, a close association between Id-1 expression and invasion has been demonstrated in both normal and cancer cells (Desprez et al., 1998; Takai et al., 2001; Lin et al., 2000).

Since the mechanism by which *R. crenulata* blocks invasion, sensitizes cells to anoikis, and inhibits tumor-sphere development did not involve EMT repression, we decided to investigate whether *R. crenulata* may be affecting the aforementioned processes by altering the expression of Id genes. Real-time PCR analysis revealed that 24 and 48 h after *R. crenulata* treatment, in both TERT-siSFRP1 and MDA-MB-231 cells, Id-1 and Id-3 mRNA levels were remarkably diminished when compared with vehicle treated cells. The finding that both Ids were similarly regulated by *R. crenulata* was not surprising considering that Id-1 and Id-3 have extensive sequence homology and similar patterns of expression during embryogenesis and in mouse adult tissues (Ellmeier and Weith, 1995). Interestingly, Shuno et. al recently showed that when Id-1 and Id-3 were stably knocked down in human pancreatic cells, proliferation and migration are significantly reduced *in vitro* and peritoneal metastases are significantly blocked *in vivo* (Shuno et al., 2008*)*. These data support the notion that *R. crenulata* may be diminishing the malignant and stem cell characteristics of TERT-siSFRP1 and MDA-MB-231 cells by reducing the expression of Id-1 and Id-3.

In contrast with Id-1 and Id-3, *R. crenulata* significantly increased the expression of Id-2 mRNA in both TERT-siSFRP1 and MDA-MB-231 cells (Figure 4). Interestingly, unlike Id-1 and Id-3, the expression of Id-2 is up-regulated *in vitro* and *in vivo* as mammary epithelial cells lose their ability to proliferate and begin the process of differentiation (Parrinello et al., 2001). Moreover, Ithana et al*.* demonstrated that Id-2 is highly expressed in well differentiated human breast cancer cells as compared to very aggressive metastatic cells. Also, in comparison to *in situ* carcinomas, Id-2 protein expression is markedly reduced in human biopsies from aggressive and invasive carcinomas. These researchers demonstrated that re-introduction of Id-2 into metastatic breast cancer cells, blocks their invasive phenotype (Itahana et al., 2003). Taking together, previous evidence and our current results suggest that *R. crenulata*, via the downregulation of Id-1 and -3 and the upregulation of Id-2 expression may, at least partially, inhibit cellular migration and invasion.

Conclusion

In this study, we show that several important cellular processes of mammary cancer cells, that are consistent with stem cell behavior, are drastically inhibited by an extract of *R. crenulata*. This medicinal plant extract inhibits migration, invasion, resistance to anoikis and tumor-sphere formation. Moreover, we propose that one mechanism by which *R. crenulata* exerts such drastic effects on these particular actions is by regulating the transcription of Id genes, a family proteins known to play a role in signaling pathways involved in differentiation, cell cycle, stem cell maintenance and tumorigenesis. Although further studies are required to define the pathways affected by the *R. crenulata*, the extract and compounds of *R. crenulata* may prove to be an excellent candidate for the treatment of aggressive breast tumors and the prevention of secondary metastases.

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