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Glyceollins as novel targeted therapeutic for the treatment of triple-negative breast cancer

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Abstract. The purpose of this study was to investigate the effects of glyceollins on the suppression of tumorigenesis in triple-negative breast carcinoma cell lines. We further explored the effects of glyceollins on microRNA and protein expression in MDA-MB-231 cells. Triple-negative (ER-, PgR- and Her2/neu-) breast carcinoma cells were used to test the effects of glyceollins on tumorigenesis *in vivo*. Following this procedure, unbiased microarray analysis of microRNA expression was performed. Additionally, we examined the changes in the proteome induced by glyceollins in the MDA-MB-231 cells. Tumorigenesis studies revealed a modest suppression of MDA-MB-231 and MDA-MB-468 cell tumor growth *in vivo*. In response to glyceollins we observed a distinct change in microRNA expression profiles and proteomes of the triple-negative breast carcinoma cell line, MDA-MB-231. Our results demonstrated that the glyceollins, previously described as anti-estrogenic agents, also exert antitumor activity in triple-negative breast carcinoma cell systems. This activity correlates with the glyceollin alteration of microRNA and proteomic expression profiles.

Introduction

Breast cancer afflicts approximately 1 in 8 women and is a leading cause of cancer-related mortality. Expression profiles of breast cancer exhibit a systematic variation and allow for the classification of breast cancer into five main groups: two estrogen receptor (ER)-positive (luminal A and B) and three ER-negative groups (normal breast-like, HER2-positive, and 'basal-like') (1). The term 'triple-negative breast cancer' (TNBC) represents a heterogeneous group of diseases and clearly does not comprise a 'single entity' (1). Although triple-negative cancer is not a synonym for basal-like cancer, basal-like cancers are preferentially negative for ER and progesterone receptor (PR) and lack HER2 expression (1). Basal-like breast carcinomas consistently express genes generally found in normal basal/myoepithelial cells of the breast, including high-molecular-weight 'basal' cytokeratins (CK; CK5/6, CK14 and CK17), vimentin, p-cadherin, αB crystallin, fascin and caveolins 1 and 2 (1). While it is clear that not all TNBC cases are characterized by the basal-like phenotype and *vice versa*, microarray-based expression analysis has demonstrated a great deal of overlap (1,2). Clinical similarities also exist between triple-negative tumors and basal-like tumors, including a higher prevalence in African-American women, more frequent incidence in younger patients, and greater aggressiveness than other molecular subgroups (1,3,4).

Of all breast cancers diagnosed approximately 75-80% are positive for ER and/or PR expression and 15-20% are positive for Her2/neu (5). Although these subtypes of disease are potentially susceptible to endocrine therapy and targeted therapy, such as trastuzumab, the remaining 10-15% of breast cancers diagnosed as triple-negative [ER(-), PR(-) and Her2/neu(-)] do not have defined therapeutic targets (6). TNBC has an aggressive clinical history as is evident by its rapid progression to a metastatic phenotype as well as a shorter time to death from distant recurrence as compared to ER(+) disease (1). It is therefore critical to identify novel targets in this disease entity.

The flavonoid family of phytochemicals, particularly those derived from soy, has received attention regarding their estrogenic activity as well as their effects on human health and disease (7-10). The observation that soy phytochemicals decrease the risk of breast cancer indicates a potential for the anti-tumorigenic activity of these compounds (11-13). Additionally, the ability of soy isoflavonoids to prevent carcinogen-induced mammary tumorigenesis further indicates the potential anti-tumorigenic effects of these compounds (14-16). Notably, the amount and type of isoflavonoid present in soy can be readily altered in response to external stimuli (17-20). We previously described an increased biosynthesis of

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the isoflavonoid phytoalexin compounds, glyceollins I, II and III, in soy plants grown under stressed conditions (elicited soy) (17,19,21).

We showed that glyceollins suppress the tumorigenesis of ER(+) and estrogen-dependent breast cancer systems, demonstrating a clear *in vivo* anti-estrogenic activity of glyceollins (22). Notably, during these studies we noted that in the absence of estrogen, glyceollin-treated tumors were significantly smaller than their negative control counterparts by day 14. This indicated that in addition to their anti-estrogenic activity, glyceollins may target ER-independent mechanisms regulating tumor cell proliferation and/or survival. In the present study, we evaluated from a biological approach the efficacy of glyceollins on TNBC tumorigenesis in immunocompromised Nu/Nu female mice. Additionally, we investigated the effects of glyceollins on microRNA (miR) expression in the triple-negative setting. In this study, we aimed to demonstrate that glyceollins act as a novel therapeutic agent in TNBC-suppressing tumorigenesis, regulating the expression of miR and altering the proteome of MDA-MB-231 cells.

Materials and methods

Cells and reagents. The MDA-MB-231 and MDA-MB-468 cell lines (human breast cancer negative for ER, PR and Her2/neu) were acquired from the American Type Culture Collection (Manassas, VA, USA) and cultured as previously described (22-24). Glyceollin mixture was isolated as previously described (22).

Xenograft model of tumorigenesis. Nu/Nu immunocompromised female mice (4-6 weeks old) were obtained from Charles River Laboratories (Wilmington, MA, USA). The animals were allowed a period of adaptation in a sterile and pathogen-free environment with food and water *ad libitum*. Mice were divided into treatment groups of 5 mice each: MDA-MB-231 + dimethyl sulfoxide (DMSO), MDA-MB-231 + glyceollins, MDA-MB-468 + DMSO, MDA-MB-468 + glyceollins. MDA-MB-231 and MDA-MB-468 cells were harvested in the exponential growth phase using a phosphate-buffered saline (PBS)/EDTA solution and washed. Viable cells ($5x10^6$) in 50 μl of sterile PBS suspension were mixed with 100 μl Reduced Growth Factor Matrigel (BD Biosciences, Bedford, MA, USA). Cells were injected bilaterally into the mammary fat pad using 27½ gauge sterile syringes. All procedures in animals were carried out under anesthesia using a mix of isofluorane and oxygen delivered by mask. Drug treatment (50 mg/kg/day glyceollins in DMSO/PBS) or vehicle (DMSO/PBS) injections were administered intraperitoneally daily for 14 days after palpable tumors had formed (MDA-MB-231, day 10; MDA-MB-468, day 25).

Tumor size was measured every 2-3 days using digital calipers. The volume of the tumor was calculated using the formula: $4/3\pi$ LS2 (L = larger radius; S = shorter radius). At necroscopy animals were sacrificed by cervical dislocation after exposure to CO_2. Tumors, uteri, livers, and lungs were removed and frozen in liquid nitrogen or fixed in 10% formalin for further analysis. All procedures involving these animals were conducted in compliance with State and Federal laws, standards of the US Department of Health and Human Services, and guidelines established by Tulane University Animal Care and Use Committee. The facilities and laboratory animals program of Tulane University are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

miR microarray. MDA-MD-231 cells were plated at a density of 2 million cells in 25 cm^2 flasks in normal culture media (Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, 1% essential amino acids, 1% non-essential amino acids and 1% sodium pyruvate) and allowed to adhere overnight at 37˚C, 5% CO_2 and air. Cells were treated with glyceollins (10 μM) or DMSO for 18 h. Cells were harvested in PBS and collected by centrifugation, and total RNA was extracted using the miRNeasy kit (Qiagen) according to manufacturer's protocol. Enrichment for miRNA was not performed. Quantity and quality of RNA was determined by absorbance (260 and 280 nm). Microarray assay was performed by LC Sciences (Houston, TX, USA). The assay started from 5 μg total RNA sample, which was size fractionated using a YM-100 Microcon centrifugal filter (Millipore, Billerica, MA, USA) and the small RNAs (<300 nt) isolated were 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining; two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was performed overnight on a μParaflo microfluidic chip using a micro-circulation pump (Atactic Technologies, Houston, TX, USA) (25,26). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target miR (from miRBase, http://microrna.sanger.ac.uk/sequences/) or other RNA (control or customer defined sequences) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were rendered by *in situ* synthesis using PGR (photogenerated reagent) chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 μl 6X SSPE buffer (0.90 M NaCl, 60 mM Na_2HPO_4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34˚C. After RNA hybridization, tag-conjugating Cy3 and Cy5 dyes were circulated through the microfluidic chip for dye staining. Fluorescence images were collected using a laser scanner (GenePix 4000B, Molecular Device, Sunnyvale, CA, USA) and digitized using Array-Pro image analysis software (Media Cybernetics, Bethesda, MD, USA). Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS (Locally-weighted Regression) filter (27). For two color experiments, the ratio of the two sets of detected signals (log2 transformed, balanced) and p-values of the t-test were calculated; differentially detected signals were those with p-values of <0.01. The array was performed using quadruplicate biological repeats. Full array data are available in Table I.

Proteomics analysis. MDA-MB-231 cells were treated with DMSO or glyceollin-mix (10 μM) for 18 h. Cells were harvested and run using 2D-electrophoresis. The first dimensional electrophoresis was performed using a Protean IEF cell unit (BioRad, Hercules, CA, USA). Precast 11-cm IPG strips with a pH range of 5-8 were used to separate the proteins based

Table I. microRNA microarray results for MDA-MB-231 cells treated with glyceollin (10 μM) for 18 h.

No.	Reporter name	p-value	Group 1 Control Mean	Group 2 Glyceollin Mean	Log2 (G2/G1)
108	hsa-miR-1268	1.09E-05	1,732	918	-0.92
156	hsa-miR-130a	3.61E-05	1,711	2,904	0.76
844	hsa-miR-940	4.32E-05	204	730	1.84
287	hsa-miR-197	4.59E-05	2,018	1,015	-0.99
277	hsa-miR-193a-5p	5.45E-05	1,452	866	-0.75
332	hsa-miR-22	8.15E-05	3,807	5,788	0.60
372	hsa-miR-29b	1.53E-04	2,616	7,692	1.56
351	hsa-miR-25	1.68E-04	6,161	4,963	-0.31
808	hsa-miR-877	1.75E-04	1,424	708	-1.01
294	hsa-miR-19b	2.30E-04	3,160	4,253	0.43
345	hsa-miR-23a*	2.33E-04	1,205	669	-0.85
828	hsa-miR-923	2.80E-04	1,743	1,243	-0.49
748	hsa-miR-638	3.58E-04	2,729	3,946	0.53
621	hsa-miR-542-5p	3.66E-04	430	142	-1.60
853	hsa-miR-99a	4.18E-04	13,257	16,348	0.30
246	hsa-miR-185	4.43E-04	1,576	1,182	-0.41
446	hsa-miR-361-5p	4.92E-04	4,553	3,574	-0.35
378	hsa-miR-301a	5.19E-04	1,176	1,436	0.29
95	hsa-miR-125b	8.65E-04	26,192	23,016	-0.19
375	hsa-miR-29c	1.03E-03	2,157	4,445	1.04
830	hsa-miR-92a	1.04E-03	10,508	9,547	-0.14
789	hsa-miR-720	1.04E-03	2,194	1,622	-0.44
238	hsa-miR-182	1.17E-03	2,216	1,493	-0.57
492	hsa-miR-423-5p	1.22E-03	5,631	3,810	-0.56
78	hsa-miR-1246	1.28E-03	561	368	-0.61
774	hsa-miR-663	1.49E-03	1,023	2,102	1.04
282	hsa-miR-195	1.62E-03	217	543	1.33
32	hsa-miR-10a	2.23E-03	1,375	762	-0.85
513	hsa-miR-454	2.58E-03	1,855	1,070	-0.79
235	hsa-miR-181c	2.64E-03	892	1,806	1.02
212	hsa-miR-151-5p	2.96E-03	8,728	7,418	-0.23
272	hsa-miR-1915	3.17E-03	1,135	1,470	0.37
407	hsa-miR-320c	3.31E-03	9,712	8,731	-0.15
397	hsa-miR-30d	3.51E-03	5,669	6,753	0.25
176	hsa-miR-138	3.83E-03	3,588	4,796	0.42
315	hsa-miR-21	3.96E-03	21,133	18,983	-0.15
211	hsa-miR-151-3p	4.09E-03	3,811	2,996	-0.35
515	hsa-miR-455-3p	4.21E-03	1,666	1,068	-0.64
523	hsa-miR-486-5p	4.50E-03	777	389	-1.00
237	hsa-miR-181d	4.51E-03	540	2,691	2.32
363	hsa-miR-28-5p	5.90E-03	983	1,137	0.21
391	hsa-miR-30a*	5.98E-03	1,452	1,015	-0.52
439	hsa-miR-34a	6.31E-03	1,307	1,644	0.33
31	hsa-miR-107	6.42E-03	7,287	8,200	0.17
356	hsa-miR-26b	6.45E-03	3,013	3,902	0.37
405	hsa-miR-320a	6.58E-03	10,409	9,155	-0.19
124	hsa-miR-1280	7.49E-03	4,155	5,245	0.34
8	hsa-let-7d*	7.73E-03	754	497	-0.60

Table I. Continued.

38	hsa-miR-1180	8.08E-03	984	501	-0.97
292	hsa-miR-19a	8.08E-03	208	602	1.54
343	hsa-miR-224	8.83E-03	2,601	1,362	-0.93
686	hsa-miR-584	9.53E-03	2,478	1,818	-0.45
	The following transcripts are statistically significant but have low signals (signal <500)				
365	hsa-miR-296-5p	9.44E-04	139	304	1.13
125	hsa-miR-1281	3.24E-03	205	440	1.10
352	hsa-miR-25*	3.32E-03	152	74	-1.04
528	hsa-miR-489	4.29E-03	460	183	-1.33
269	hsa-miR-1913	4.37E-03	126	288	1.19
553	hsa-miR-505*	5.53E-03	340	130	-1.39
464	hsa-miR-374b	6.50E-03	201	282	0.49
778	hsa-miR-665	8.19E-03	55	160	1.54
245	hsa-miR-184	9.20E-03	274	36	-2.93

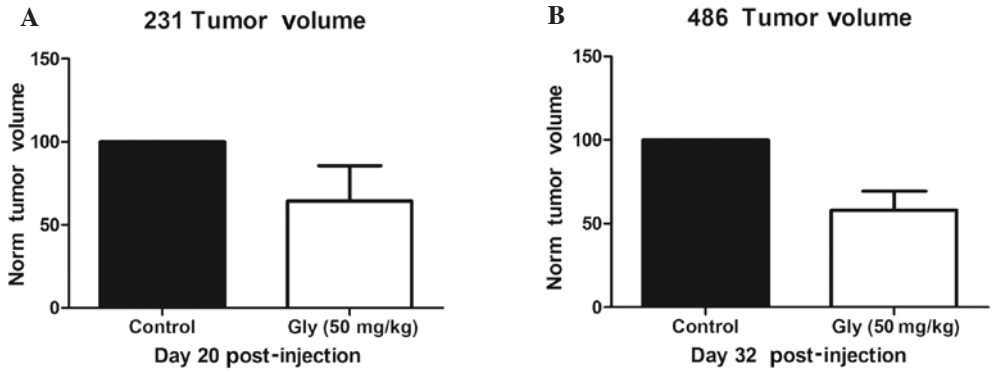

Figure 1. Glyceollin decreases tumorigenesis of triple-negative breast carcinoma *in vivo*. Nu/Nu female mice (4-6 weeks old) were injected in the MFP with $5x10^6$ (A) MDA-MB-231 or (B) MDA-MB-468 cells. After tumor formation (10 and 25 days, respectively) mice were administered daily intraperitoneal injections of vehicle or glyceollins (50 mg/kg) for (A) 9 or (B) 7 days. Tumors were measured via digital caliper. Bars are the mean tumor volume at endpoint (normalized to vehicle) ± SEM.

on their isoelectric pH. The second dimensional electrophoresis was carried out in a BioRad Criterion electrophoresis cell system. Stained gels were scanned with a Gel Doc-XR image system (BioRad) and analyzed with the PDQuest software (version 8.01). The proteins of interest were marked for excision and excised from gels using a Quest Spot cutter (BioRad), digested and analyzed by an LC-Nanospray-MS system. The tandem MS spectra were analyzed against the ipi.human.v3.27 database using SEQUEST software and tabulated.

Statistical analysis. Studies were analyzed by the unpaired Student's t-test (Graph Pad Prism V.4) and p-values of <0.05 were considered statistically significant.

Results and Discussion

Glyceollins partially suppress growth of triple-negative breast tumor growth in vivo. To determine the therapeutic relevance of glyceollins in the triple-negative setting, MDA-MB-231 and MDA-MB-468 cells were used in an *in vivo* xenograft model of tumorigenesis. Immunocompromised female nude mice were injected in the mammary fat pad (MFP) with either MDA-MB-231 (Fig. 1A) or MDA-MB-468 (Fig. 1B) cells mixed with reduced growth factor matrigel. After palpable tumor formation (MDA-MB-231, day 10; MDA-MB-468, day 25), mice were randomized into treatment groups (n=5) and treated with vehicle or glyceollins (50 mg/kg/day). Tumor volume of MDA-MB-231 and MDA-MB-468 cells treated with glyceollins showed decreased tumor growth compared to vehicle-treated control tumors at endpoint analysis (Fig. 1, 64.36±21.29 mm^3 and 58.16±11.28 mm^3, respectively). These results demonstrate the tumor-suppressive effects of glyceollins on triple-negative breast carcinoma cell lines and indicate the clinical significance and therapeutic potential of glyceollins in the TNBC.

Glyceollins alter the miRnome of triple-negative breast carcinoma cells consistent with tumor-suppressive effects. Altered miR expression is common among a number of types of cancer and this dysregulation is known to promote

tumorigenesis, hormone independence and drug resistance, epithelial-mesenchymal transition (EMT) and metastasis (28-39). miR microarray analysis of MDA-MB-231 cells treated with glyceollins for 18 h revealed a number of changes in the miRNA expression profile compared to vehicle treated cells. Fig. 2 shows a heat map of miR expression changes for 4 independent samples. Tables II and III show the miRs found to have a significantly altered expression (increased or decreased, respectively) in response to treatment with glyceollins ($p<0.01$). A number of the miRs demonstrating a significantly increased expression following treatment with glyceollins have been characterized as tumor suppressers inhibiting cell cycle and proliferation (miR-181c/d), EMT and metastasis (miR-22, 29b/c, 30d, 34a, 195), or directly targeting known oncogenes (miR-26b). Those miRs with a significantly decreased expression induced by glyceollins have been identified as oncomiRs with roles in promoting tumorigenesis (miR-21, 193-5p) and metastasis (miR-185, 224).

Among the most highly expressed miRs following treatment with glyceollins were miR-19a/b, 22, 29b/c, 181c/d, 195, 663 and 940. Notably, a number of the miRs that demonstrated glyceollin-induced expression have previously been documented as having tumor-suppressive effects. For example, miR-22 has been classified as a tumor-suppressive miR in metastatic breast cancers, as it has been shown to target oncogenes EVI-1, ERBB3 and CDC25C (40), as well the pro-metastatic gene EZR in ovarian cancer (41,42). miR-26b inhibits glioma tumor cell proliferation, survival, and migration by directly targeting EPHA2 (43). Further evidence of the tumor-suppressive nature of miR-26b include its ability to induce apoptosis via repression of SLC7A11 and the decreased expression of miR-26b in breast carcinoma patient samples (44).

miR-29b/c have been shown to directly inhibit the cell cycle transcription factor MYBL2 and in turn induce tumor cell senescence (45). miR-29 also plays a role in maintaining adequate cell adhesion by regulating extracellular matrix proteins (46) including collagens (47,48) and elastin (49), and regulates cell survival by targeting the anti-apoptotic MCL1 (50). Furthermore, miR-29 has been shown to induce expression of the tumor suppressor p53 by inhibiting the Rho-GTPase CDC42 (51).

Decreased expression of miR-181c due to hypermethylation has been observed in gastric carcinoma and its targets include the oncogenes NOTCH4 and KRAS (52). Additionally, miR-195 expression is significantly decreased in breast carcinoma patient samples (53), and a decreased expression of miR-195 has been correlated with decreased survival and increased metastasis in colorectal cancers (54). Direct targets of miR-195 include the oncogene RAF1, cell cycle regulators CCND1 (53) and CCNE1 (55), as well as the anti-apoptotic BCL2 (56).

The remaining two miRs with fold changes >2 have also been found to play anti-tumorigenic roles in cancer. miR-663 inhibits AP-1 activity by directly targeting JunB and JunD (57) and has been described as a tumor suppressor in gastric cancer (58). *In silico* predicted targets (TargetScan and miRANDA) of miR-940 include RhoA, a prominent mediator of invasion and metastasis.

Notably, although miR-19 is often referred to as an oncomiR due to its inclusion in the miR-17-92 oncogenic cluster,

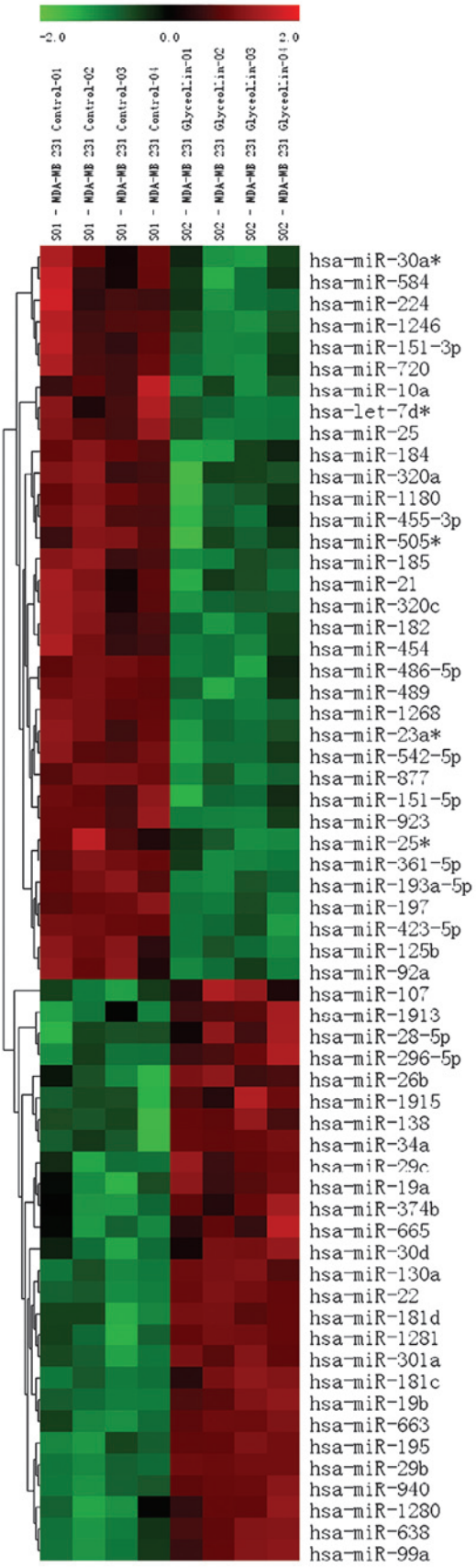

Figure 2. Glyceollins regulation of microRNA expression in MDA-MB-231 cells. Heatmap of microRNA changes induced by treatment with glyceollins (10 μM) after 18 h in MDA-MB-231 cells. microRNAs demonstrating statistically significant changes in expression are shown ($p<0.01$). Green indicates down-regulated expression and red indicates up-regulated expression of microRNAs. Individual samples are shown in columns while specific microRNAs are indicated by rows as labeled.

Table II. microRNA with increased expression following glyceollin treatment.

miRNA	Mean fold-change	p-value	miRNA	Mean fold-change	p-value
19a	2.91	<0.01	130a	1.69	<0.001
19b	1.35	<0.001	301a	1.22	<0.001
22	1.52	<0.001	138	1.34	<0.01
26b	1.29	<0.01	181c	2.03	<0.01
			181d	4.99	<0.01
28-5p	1.16	<0.01	195	2.51	<0.01
29b	2.95	<0.001	638	1.44	<0.001
29c	2.06	<0.01			
30d	1.19	<0.01	663	2.06	<0.01
34a	1.26	<0.01	940	3.58	<0.001
99a	1.23	<0.001	1280	1.27	<0.01
107	1.13	<0.01	1915	1.29	<0.01

Table III. microRNA with decreased expression following glyceollin treatment.

miRNA	Mean fold-change	p-value	miRNA	Mean fold-change	p-value
10a	-1.80	<0.01	361-5p	-1.27	<0.001
21	-1.11	<0.01	423-5p	-1.47	<0.01
23a*	-1.80	<0.001	454	-1.73	<0.01
25	-1.24	<0.001	455-3p	-1.56	<0.01
30a*	-1.43	<0.01	486-5p	-2.00	<0.01
92a	-1.10	<0.01	542-5p	-3.03	<0.001
125b	-1.14	<0.001	584	-1.37	<0.01
151-3p	-1.27	<0.01	720	-1.36	<0.01
151-5p	-1.17	<0.01			
182	-1.48	<0.01	877	-2.01	<0.001
185	-1.33	<0.001	923	-1.40	<0.001
193a-5p	-1.68	<0.001	1180	-1.96	<0.01
197	-1.99	<0.001	1246	-1.53	<0.01
224	-1.91	<0.01	1268	-1.89	<0.001
320a	-1.14	<0.01	let-7d[a]	-1.52	<0.01
320c	-1.11	<0.01			

Zhang *et al* have recently demonstrated the ability of miR-19 to directly target tissue factor (TF), a known promoter of cancer cell survival, angiogenesis, and metastasis (59). Therefore, miR-19 may also play a tumor-suppressive role in breast cancer.

Among the most downregulated miRs following treatment with glyceollins were 193a-5p, 197, 224, 486-5p, and 542-5p, all of which have been associated with cancer progression. For instance, miR-193a-5p has been shown to target pro-apoptotic p73 and limit the effects of chemotherapy (60), while the oncomiR miR-197 has been shown to directly target the tumor suppressor, FUS1 (61). miR-224 has been associated with cancer progression (62) and enhanced cell migration and invasion by increasing the expression of the pro-invasive PAK4 and MMP-9 (63). Additionally, miR-224 and miR-486-5p promote cell migration and invasion by targeting the tumor suppressor CD40 (64,65).

miR-542-5p expression has also been associated with maintenance of the mesenchymal phenotype (66), a key characteristic of the TNBC phenotype and driver of cell motility and invasiveness. The reversal of the mesenchymal phenotype to a more epithelial morphology through the process of mesenchymal-to-epithelial transition (MET) represents an area of high-impact research for the development of novel therapeutics.

Although not a marked change, treatment with glyceollins decreases the expression of miR-21. miR-21 is one of the most established and highly researched miRs for its oncogenic role

Table IV. Effects of glyceollins on the MDA-MB-231 cell proteome.

Spot	Gene symbol	Gene name	Mean intensity ratio	p-value
203	EEF1D	Elongation factor 1-δ	36.50	<0.01
1004	ARHGDIA	Rho GDP-dissociation inhibitor 1	115.85	<0.001
2102	CLIC1	Chloride intracellular channel protein 1	129.94	<0.001
2103	TPD52L2	Isoform 2 of tumor protein D54	43.90	<0.01
2204	EIF2S1	Eukaryotic translation initiation factor 2 subunit 1	55.62	<0.01
3103	CLIC4	Chloride intracellular channel protein 4	44.37	<0.01
5004	NME1	Non-metastatic cells 1 (NM23-H1)	24.89	<0.01
5304	GIPC1	PDZ domain-containing protein GIPC1	21.41	<0.01
5405	MAP2K2	Mitogen-activated protein kinase kinase 2	29.96	<0.01
5406	TARDBP	TAR DNA-binding protein 43	29.96	<0.01
7706	VIM	Vimentin	-13.84	<0.01
9903	DDX1	ATP-dependent RNA helicase DDX1	-34.75	<0.001
9904	KHSRP	Far upstream element-binding protein 2	-31.31	<0.01
2304	SEC13	SEC13-related protein	-10.07	<0.01
5506	HNRPH1	Heterogeneous nuclear ribonucleoprotein H1	-28.63	<0.001
7202	HNRPH3	Heterogeneous nuclear ribonucleoprotein H3	-66.35	<0.01
8404	HNRPD	Heterogeneous nuclear ribonucleoprotein D0	-15.28	<0.001
3605	FKBP4	FK506-binding protein	-17.83	<0.01

in cancer (67), and has been shown to be highly overexpressed in TNBC (68). The expression of miR-21 in breast tumors has been associated with poor prognosis (31), development of drug resistance (69,70), and increased rate of recurrence (39). Targets of miR-21 include prominent tumor suppressors PTEN (71,72) and PDCD4 (73), as well as inhibitors of metastasis, such as TIMP3 (74) and TPM1 (75,76).

The function of the remaining two miRs downregulated more than 1.95-fold by glyceollins, miR-877 and 1180, has yet to be determined at the time of this publication. Although the function of these miRs is not currently known, putative targets predicted by TargetScan include p53 inducible nuclear protein 2 (TP53INP2) and the cell cycle regulator, CDC40 (putative targets for miR-877); a regulator of cell adhesion, PUNC, the pro-apoptotic gene, BAD and BAMBI, a negative regulator of TGFβ known to mediate cell transformation (putative targets of miR-1180).

Glyceollins alter the proteome of MDA-MB-231 breast carcinoma cells in a manner indicative of tumor suppressive effects. The treatment of MDA-MB-231 cells with glyceollins for 18 h generated distinct protein spot patterns as analyzed by 2D-gel electrophoresis. Sequence analysis of selected spots revealed a number of proteins up- and downregulated by glyceollins (Table IV). While each of these spots represents a target for validation and mechanistic analysis, two proteins identified are known to play key roles in breast cancer tumorigenesis and progression. We observed an almost 25-fold upregulation of NME1 (NM23-H1) by glyceollin treatment. NME1 is a known metastasis suppressor gene (77) and is a putative target of two miRs with a significantly decreased expression following treatment with glyceollins, miR-486-5p and miR-542-5p. Notably, as mentioned above, miR-542-5p expression has been linked to the maintenance of the mensenchymal phenotype. Decreased expression of miR-542-5p, as well as an increased expression of NME1, indicates a reversal of EMT and a suppression of metastasis by glyceollins. A second target identified via our proteomics approach is vimentin, whose expression was downregulated more than 13-fold by glyceollins. Vimentin is a marker for epithelial-to-mesenchymal transition and is highly expressed in numerous TNBCs and cell lines including MDA-MB-231 (78). Vimentin is a proven target of miR-30d (79) and a predicted target of miR-138, both found to be significantly increased by glyceollins. These data suggest that the effects of glyceollins on TNBC cell lines are achieved via regulation of miRs, which in turn regulate known oncogenes and tumor suppressors. Taken together, these data indicate that treatment of TNBC cells with glyceollins inhibit tumorigenesis and induce a miR expression profile correlative to a less aggressive phenotype.

In conclusion, the results from our study demonstrate the ability of glyceollins to inhibit tumor growth of the triple-negative breast carcinoma cell lines, MDA-MB-231 and MDA-MB-468. Furthermore, it is known that the dysregulation of miR expression is a characteristic of numerous cancer cancer types including breast carcinoma. miR microarray analysis of MDA-MB-231 cells treated with glyceollins revealed significant alterations of the miR expression profile consistent with a less aggressive phenotype.

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