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MicroRNA-93 regulates NRF2 expression and is associated with breast carcinogenesis

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MicroRNAs (miRNA) are small non-coding RNAs that regulate the expression of approximately 60% of all human genes and play important roles in disease processes. Recent studies have demonstrated a link between dysregulated expression of miRNAs and breast carcinogenesis. Long-term estrogen exposure is implicated in development of human breast cancers, yet underlying mechanisms remain elusive. We have recently demonstrated that antioxidant vitamin C (vit C) prevents estrogen-induced breast tumor development. In this study, we investigated the role of vit C in the regulation of microRNA-93 (miR-93) and its target gene(s) in a rat model of mammary carcinogenesis. Female August Copenhagen Irish (ACI) rats were treated with vit C in the presence or absence of 17β-estradiol (E2) for 8 months. We demonstrate an increased expression of the miR-93 in E2-treated mammary tissues and in human breast cell lines and vit C treatment reverted E2-mediated increase in miR-93 levels. MiRNA target prediction programs suggest one of the target genes of miR-93 to be nuclear factor erythroid 2-related factor 2 (NRF2). In contrast with miR-93 expression, NRF2 protein expression was significantly decreased in E2-treated mammary tissues, mammary tumors, and in breast cancer cell lines, and its expression was significantly increased after vit C treatment. Ectopic expression of miR-93 decreased protein expression of NRF2 and NRF2-regulated genes. Furthermore, miR-93 decreased apoptosis, increased colony formation, mammosphere formation, cell migration and DNA damage in breast epithelial cells, whereas silencing of miR-93 in these cells inhibited these carcinogenic processes. Taken together, our findings suggest an oncogenic potential of miR-93 during E2-induced breast carcinogenesis.

Introduction

A growing body of clinical and epidemiological literature supports a role for estrogens in human breast carcinogenesis (1–7). Two-thirds of human breast cancers share a common feature in that they are estrogen dependent (8). However, our understanding of the molecular mechanisms underlying the initiation and progression of estrogen-associated breast cancers is rather poor and are being investigated in rodent models and in relevant breast cell lines. The female August Copenhagen Irish (ACI) rat model is widely used as an accepted rodent model of breast cancer to understand the paradigms of human breast carcinogenesis as tumors in this model share many features with human breast cancers, e.g. estrogen dependence, aneuploidy and genomic instability (9–11). Published data from our laboratory and that of others suggest an important role of oxidative stress in estrogen-induced breast carcinogenesis (1,2,7,12,13). We have recently reported that antioxidants vitamin C (vit C) or butylated hydroxyanisole can drastically inhibit 17β-estradiol (E2)-induced breast cancer in the rat model (2,7). In the same animal model, we have demonstrated that antioxidant gene-regulating transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) is significantly decreased during E2-induced breast carcinogenesis and that vit C may prevent E2-induced breast cancer *via* induction of NRF2 (5). However, the mechanism of regulation of NRF2 during estrogen-induced breast cancer is not known. In this study, we investigated the role microRNA-93 (miR-93) plays in regulation of NRF2 and in estrogen-dependent breast carcinogenesis *in vivo* in a rat model of estrogen-induced breast cancer and *in vitro* using human breast cancer and non-neoplastic breast epithelial cell lines.

MicroRNAs (miRNAs) are endogenous, small non-coding RNAs, which are involved in post-transcriptional control of gene expression (14,15). These small RNAs are assumed to directly control the expression of approximately 60% of the human genome and are involved in the regulation of many cellular activities, such as metabolism, development, proliferation, differentiation and apoptosis (15,16). MiRNAs are frequently dysregulated in human cancers and can act either as potent oncogenes or as tumor suppressor genes (17,18).

In this study, we demonstrated that E2 treatment induced miR-93 expression in mammary and mammary tumor tissues, whereas vit C treatment inhibited E2-mediated upregulation of its expression in mammary tissues. MiR-93 was able to regulate oncogenic process in mammary through regulation of its target gene *NRF2*. Therefore, this study presents significant insight into the role of miR-93 and its target gene *NRF2* in E2-induced breast cancer and its prevention by antioxidant vit C.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; E2, 17β-estradiol; miR-93, microRNA-93; miRNA, microRNA; mRNA, messenger RNA; NRF2, nuclear factor erythroid 2-related factor 2; 8-OHdG, 8-hydroxydeoxyguanosine; PBS, phosphate-buffered saline; ROS, reactive oxygen species; siRNAs, small interfering RNAs; vit C, vitamin C.

Materials and methods

Treatment of animals

Female ACI rats (4 weeks of age; Harlan Sprague Dawley, Indianapolis, IN) were housed under controlled temperature, humidity and lighting conditions. After a 1-week acclimatization period, rats were divided into following different groups: control, E2, vit C and vit C + E2. Rats were implanted subcutaneously with 3 mg of E2 pellets. E2 pellets were prepared in 17 mg cholesterol as a binder as described previously (19). Control and vit C groups of rats received 17 mg cholesterol pellet only. Vit C (1%) was administered in drinking water. All animals were fed phytoestrogen-free AIN76A diet (Dyets, Bethlehem, PA). All animals underwent treatments as described above for 240 days. At the end of the experimental time period, animals were anesthetized using isoflurane and euthanized. Mammary tissues (both tumor and normal) were removed and snap frozen in liquid nitrogen for further analyses. The animals were treated and handled according to the guidelines of the University of Missouri Animal Care and Use Committee. Animal protocols used in this study were approved by the institutional animal care and use committee (approval number AC-AAAA9396).

Cell culture and transfection studies

Non-tumorigenic human breast epithelial cell line MCF-10A and human breast neoplastic cell line T47D were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (50:50) media (Mediatech, Herndon, VA). Twenty-four hours before treatment, cells were washed twice with phosphate-buffered saline (PBS) and then grown in phenol red-free DMEM/F12 (50:50) media supplemented with charcoal–dextran-stripped serum. Cells were treated with E2 (10 or 50 nM), vit C (1 mM) and vit C + E2 for up to 48 h. For transfection studies, small interfering RNAs (siRNAs) for NRF2 and scrambled siRNAs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). AntimiR-93, premiR-93, antimiR and premiR negative controls were purchased from Ambion (Austin, TX). MCF-10A and T47D cells were transfected with antimiR-93 (25 nmol/l) or premiR-93 (25 nmol/l) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). MCF-10A cells were also used to transfect siNRF2 (20 nmol/l) using Lipofectamine 2000 (Invitrogen). Scrambled siRNA (20 nmol/l)-, antimiR negative control- (25 nmol/l) and premiR negative control- (25 nmol/l) transfected cells were used as negative controls (5).

Real-time PCR analysis

Total RNA was isolated from ACI rat tissues and cells in culture using Tri Reagent (Molecular Research Center Inc, Cincinnati, OH) according to the supplier's recommendations. Five microgram total RNA was reverse transcribed using random hexanucleotide primers and superscript II reverse transcription system (Invitrogen). Real-time PCR by SYBR Green chemistry was performed using iCycler iQ5 system (Bio-Rad Laboratories, Hercules, CA) with rat- and human-specific *NRF2* QuantiTect primers (Invitrogen) and pri-miR-93-specific primers, which can detect pri-miR-93 in both human and rat samples. The expression of U6 small nuclear RNA and cyclophilin was used for normalization and quantification of pri-miR-93 and *NRF2* expression, respectively. Data were analyzed from at least five different animals/cell line samples from each group. Primers used were as follows: pri-U6: 5′-CTCGCTTCGGCAGCACA-3′ and 5′-AACGCTTCACGAATTTGCGT-3′; pri-miR-93 5′-AAGTGCTGTTCGTGCAGGT-3′ and 5′-CTCGGGAAGTGCTAGCTCA-3′ and cyclophilin: 5′-CCCACCGTGTTCTTCGACAT-3′ and 5′-CCAGTGCTCAGAGCACGAAA-3′.

Western blot analysis

Approximately, 50 mg of ACI rat tissues were homogenized in a tissue protein extraction buffer (Thermo Scientific, Rockford, IL). Lysates from cell lines were prepared in RIPA buffer with protease inhibitor cocktail (Sigma–Aldrich, St Louis, MO). The Pierce BCA Protein Assay kit was used to determine protein concentrations (Pierce, Rockford, IL). Approximately, 80 µg of total protein from ACI rat tissues or 30 µg of protein from cell lines in culture were size fractionated on a 12% sodium dodecyl sulfate–polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA) under standard conditions (4,5). Primary antibody against NRF2 (Santa Cruz Biotechnology) was used for immunodetection. Chemiluminescent detection was performed using the BM Chemiluminescence Detection kit (Roche, Indianapolis, IN) and Alpha Innotech FluorChem HD2 (Alpha Innotech, San Leandro, CA) gel documentation system. Membranes were reprobed with α-tubulin antibody (Santa Cruz Biotechnology). Intensities of the bands were quantified and normalized using AlphaEase FC StandAlone software (version 6.0.0.14; Alpha Innotech).

Clonogenic cell survival assay

About 500 viable MCF-10A cells transiently transfected with antimiR-93, premiR-93, antimiR negative control or premiR negative control oligonucleotides for 48 h or untransfected MCF-10A cells were seeded in six-well plates and allowed to grow for 24 h in phenol red-free complete media. The cells were then incubated in the presence or absence of E2 for 72 h, washed in PBS and incubated for an additional 8 days in complete medium. The colonies obtained were washed with PBS and fixed in 10% formalin for 10 min and again washed twice with PBS followed by staining with crystal violet (0.1% w/v solution in 10% ethanol). The colonies were counted, photographed and compared with respective untreated cells. Each treatment was done in triplicate.

Mammosphere formation assay

Mammosphere formation assay was carried out in ultra-low attachment plates (Corning, Lowell, MA). Briefly, 5000 viable MCF-10A cells transiently transfected with antimiR-93, premiR-93, antimiR negative control or premiR negative control oligonucleotides for 48 h or untransfected MCF-10A cells were seeded into a 24-well plate. Cells were grown in serum-free DMEM/F12 (50:50) medium supplemented with 1× B27 (Invitrogen), 20 ng/ml epidermal growth factor (Invitrogen), 20 ng/ml basic fibroblast growth factor (Invitrogen), 1 µg/ml hydrocortisone (BD Biosciences, Bedford, MA), 5 µg/ml insulin (Invitrogen), 0.1% penicillin/streptomycin (Lonza, Walkersville, MD) and 4 µg/ml heparin calcium salt (Thermo Scientific) at 37°C under 5% CO_2 in the presence or absence of E2 (10 nM). After 6–8 days of incubation, mammospheres were viewed under the microscope and photographed. Three replicate wells from a 24-well plate were used for each experimental condition.

Cell migration assay

Cell migration assay was used to study the metastatic potential of the cells. Briefly, a confluent monolayer of MCF-10A cells transiently transfected with antimiR-93, premiR-93, antimiR negative control or premiR negative control oligonucleotides for 48 h was established and then a scratch was made through the monolayer, using a standard 200 µl plastic pipette tip, washed twice with PBS and replaced in phenol red-free complete media. Cells migrate into the scratch area as single cells from the confluent sides. After 24 h, the width of the scratch gap was viewed/measured under the microscope and photographed. Three replicate wells from a six-well plate were used for each experimental condition.

Cell apoptosis assay

Caspase-3/7 activities were measured using the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega, Madison, WI) according to the manufacturer's protocol. MCF-10A cells transiently transfected with antimiR-93, premiR-93, antimiR negative control or premiR negative control oligonucleotides for 48 h or untransfected MCF-10A cells were seeded in triplicate in 96-well, white, flat bottom cell culture plates (Corning). After 24 h of seeding, cells were lysed with lysis buffer containing caspase substrate Z-DEVD-R110 and incubated at room temperature for 6 h. Caspase activities were measured by detection with a fluorescence microplate reader (Titertek, Huntsville, AL), and the fluorescence was measured at an excitation/emission wavelength of 485/535 nm.

8-OHdG estimation assay

8-Hydroxydeoxyguanosine (8-OHdG), an accepted marker of oxidative stress-mediated DNA damage, was estimated in antimiR-93-, premiR-93-, antimiR negative control- or premiR negative control-transfected MCF-10A cells and E2-treated MCF-10A cells using Oxiselect oxidative DNA damage ELISA kit (Cell Biolabs, San Diego, CA) as described previously (5).

Statistical analyses

Statistical analyses were performed by using Sigma Plot 11.0 (Systat Software, San Jose, CA) and IBM SPSS Statistics 19 software (IBM Inc, Armonk, NY). The unpaired *t*-test analysis was used to calculate *P* values for comparisons of NRF2 messenger RNA (mRNA) and protein levels, and miR-93 expression levels between treated animals and respective age-matched controls, as well in cell lines. The unpaired *t*-test analysis was also used to calculate *P* values for comparisons of colony and mammosphere formation, cell migration, caspase-3/7 activity and 8-OHdG levels in E2-treated, antimiR-93- or premiR-93-transfected cells and respective controls. Fisher's exact test was used to compare tumor incidence between two treatment groups. A P value <0.05 was considered significant.

Results

Estrogen treatment upregulates whereas vit C inhibits E2-mediated upregulation of miR-93 in mammary tissues and breast cell lines

Our preliminary miRNA array data (Singh *et al.*, unpublished data) revealed significant upregulation of miR-93 in E2-induced rat mammary tumors compared with age-matched control mammary tissues. We then validated our miRNA array data and reconfirmed estrogen-mediated regulation of miR-93 in non-neoplastic breast epithelial cell line MCF-10A, in breast cancer tumor cell line T47D, in rat mammary tissues and in E2-induced breast tumors, by quantitative real-time PCR. Real-time PCR data demonstrated that E2 treatment significantly increased expression of miR-93 in rat mammary tissues (~1.7-fold), mammary tumors (~1.9-fold), human normal (non-neoplastic) breast epithelial cell line MCF-10A (~1.7-fold) and in human neoplastic breast epithelial cell line T47D (~1.7-fold) compared with respective controls (Figure 1 and Table I). Because estrogen-metabolism-mediated oxidative stress is implicated in breast carcinogenesis (1,2,7) and antioxidant vit C inhibits estrogen-induced breast cancer (2), we also examined the effect of vit C on miR-93 expression. We determined the expression of miR-93 in rat mammary tissues as well as in MCF-10A and T47D cells after vit C treatment in the presence or absence of E2. Although vit C treatment showed a trend toward decrease in miR-93 levels in both *in vivo* and *in vitro* studies (Figure 1 and Table I), these changes were not statistically significant. Importantly, vit C inhibited estrogen-mediated increase in miR-93 expression, both *in vitro* and *in vivo* (Figure 1 and Table I).

Estrogen treatment downregulates whereas vit C upregulates NRF2 in mammary tissues and breast cell lines

mRNA and protein expression of NRF2 in rat mammary and mammary tumor tissues as well as in MCF-10A and T47D cells treated with E2, vit C and vit C + E2 were examined by real-time PCR and western blot analyses, respectively. A significantly decreased mRNA as well as protein expression of NRF2 in E2-treated mammary and mammary tumor tissues compared with age-matched control mammary tissues was observed (Figure 2

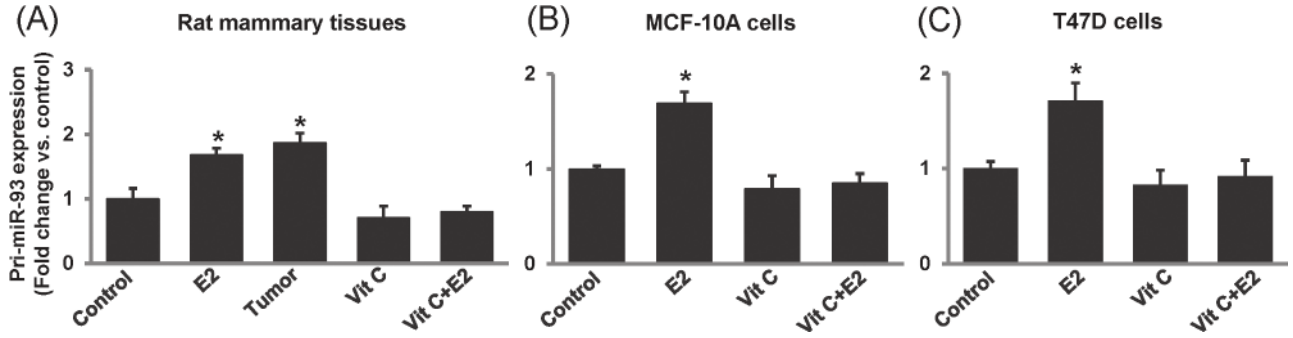

Fig. 1. Estrogen treatment upregulates, whereas vit C inhibits E2-mediated upregulation of miRNA-93 expression. Real-time reverse transcription–PCR analyses of pri-miR-93 expression in mammary tumors and in mammary tissues of rats treated with E2, vit C and vit C + E2 for 240 days (**A**), and in MCF-10A (**B**) and T47D cells (**C**) after E2 (10 nM), vit C (1 mM) and vit C + E2 treatment for 24 h. These data presented are an average of fold change values compared with respective control values obtained for at least five different samples ± standard error of the mean. Pri-miR-93 expression data were normalized to U6 small nuclear RNA as an internal control. '*' indicates a P value <0.05 compared with controls.

Table I. MiR-93 expression, NRF2 protein expression and tumor incidence in female ACI rats after different treatments

Treatment	*n*	Pri-miR-93 expression (fold change versus control)	NRF2 protein expression (fold change versus control)	Tumor incidence (%)
Control	10	1.00	1.00	0
E2	11	1.68*	0.61*	82
Tumor	11	1.87*	0.35*	—
Vit C	17	0.71	2.53*	0
Vit C + E2	17	0.80	2.36*	29**

MiR-93 expression and NRF2 protein expression levels were measured in mammary tumors and in mammary tissues from control, E2-, vit C- and vit C + E2-treated rats after 240 days of treatment. Column one lists the different treatments each group of animals received. The number of animals per group (*n*) is listed in column two. Columns three and four show the pri-miR-93 expression and NRF2 protein expression, respectively, as an average of values obtained for at least five different animals. The percent tumor incidence in each treatment group after 240 days of treatment is listed in column five.
*$P < 0.05$ compared with control tissue.
**$P < 0.05$ compared with E2-treated group.

and Table I). Vit C treatment significantly increased NRF2 mRNA and protein expression and inhibited E2-induced tumorigenesis (Figure 2 and Table I). Co-treatment with vit C + E2 prevented E2-mediated decrease of NRF2 (Figure 2 and Table I). Similarly, in *in vitro* experiments using MCF-10A and T47D cell lines, E2 treatment significantly decreased and vit C treatment alone or in combination with E2 significantly increased NRF2 mRNA and protein expression (Figure 2). Because the expression of a miRNA is inversely correlated to the protein expression of its target genes (20,21), the inverse correlation between miR-93 expression and NRF2 protein expression indicated a miR-93-mediated regulation of NRF2 (Figures 1 and 2; Table I).

NRF2 is a potential target of miR-93

miRNA target prediction using 'miRanda' miRNA target prediction program revealed *NRF2* as one of the possible target gene of miR-93 (Figure 3A) (microRNA.org). NRF2 is a known regulator of antioxidant status in biological system (5,22,23) and we have earlier reported that E2-metabolism-mediated oxidative stress is implicated in breast carcinogenesis (1,2,7). Therefore, after analyzing miR-93 expression, we determined if *NRF2* is a potential target of miR-93. We silenced or overexpressed miR-93 in MCF-10A and T47D cells using antimiR and premiR for miR-93, respectively. We then examined the effect of silencing and overexpression of miR-93 on protein expression of NRF2. We demonstrated that antimiR-93-mediated silencing of miR-93 increased protein expression of NRF2 and NRF2-regulated genes, NAD(P)H:quinone oxidoreductase (5) and superoxide dismutase 3 (24), in both MCF-10A and T47D cells (Figure 3B). On the contrary, transfection of premiR-93 decreased protein expression of NRF2, NAD(P)H:quinone oxidoreductase and superoxide dismutase 3 in MCF-10A and T47D cells (Figure 3B).

miR-93 increases clonability, mammosphere formation and migratory properties of MCF-10A cells

Overexpression of miR-93 in E2-treated mammary and mammary tumor tissues indicated a possible oncogenic role of this miRNA in E2-induced breast carcinogenesis (Figure 1 and Table I). To examine the oncogenic potential of miR-93, we performed colony formation, mammosphere formation and cell migration assays in MCF-10A cells after premiR-mediated overexpression of miR-93. Significantly increased colony formation, mammosphere formation and cell migration in premiR-93-transfected MCF-10A cells compared with premiR control-transfected or vehicle-treated MCF-10A cells suggested the carcinogenic potential of miR-93 (Figure 4).

To further confirm whether silencing of miR-93 inhibits clonability, migratory and mammosphere formation potential, we silenced miR-93 in MCF-10A cells using antimiR-93 and all the assays as discussed above were carried out. We show that silencing of miR-93 significantly decreased colony formation, mammosphere formation and cell migration capability of MCF-10A cells (Figure 4).

NRF2 is known to play protective role against E2-induced breast carcinogenesis (5) and we showed that miR-93 overexpression inhibited NRF2 protein expression (Figure 3). Thus, silencing of *NRF2* in MCF-10A cells should mimic the condition of miR-93 overexpression in these cells. To compare *NRF2* silencing with miR-93 overexpression, we knocked down *NRF2* in MCF-10A cells using siRNA and then performed colony formation, mammosphere formation and cell migration assays. We demonstrated similar results with *NRF2* silencing as we did with miR-93 overexpressed MCF-10A (premiR-93-transfected) cells (Figure 4).

miR-93 inhibits apoptosis and increases oxidative DNA damage

Because we observed an oncogenic potential of miR-93, we further examined the effect of silencing or overexpression of miR-93 on

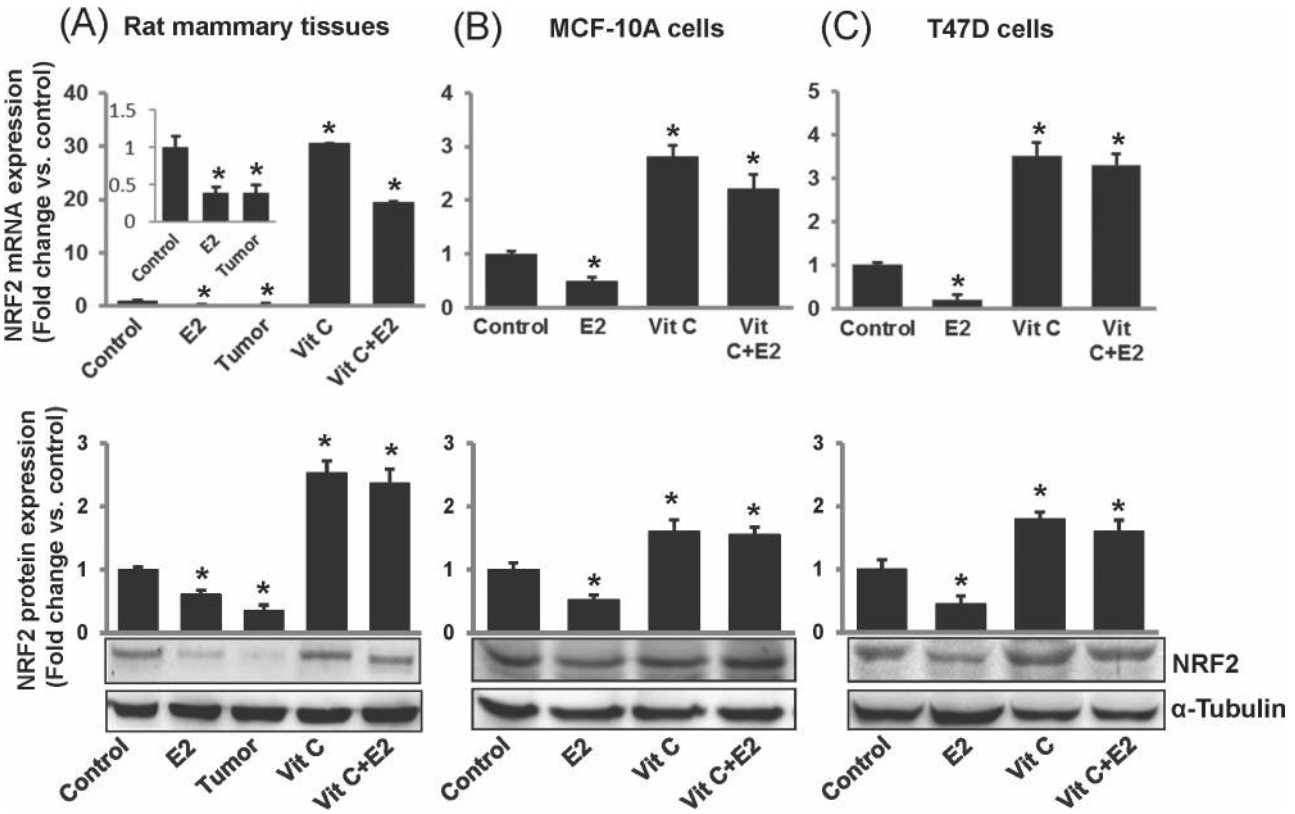

Fig. 2. Estrogen treatment downregulates, whereas vit C upregulates NRF2. Real-time reverse transcription–PCR and western blot analyses of NRF2 expression in mammary tumors and in mammary tissues of rats treated with E2, vit C and vit C + E2 for 240 days (**A**), and in MCF-10A (**B**) and T47D cells (**C**) after E2 (10 nM), vit C (1 mM) and vit C + E2 treatment for 24 h. mRNA expression data were normalized to cyclophilin as an internal control, whereas protein band intensities were quantified and normalized to α-tubulin. The bar graphs represent NRF2 mRNA and protein fold change (mean ± standard error of the mean) from at least five different replicates compared with respective control samples. '*' indicates a P value <0.05 compared with controls.

apoptosis, an important cell death mechanism, which is generally downregulated in cancers (25). Induction of caspase activity has been shown to be involved in the onset of apoptosis (26). Thus, to observe the effect of miR-93 on apoptosis, we carried out caspase-3/7 activity assay with MCF-10A cells transfected with either antimiR-93 or premiR-93. We observed a significant increase in caspase-3/7 activity in MCF-10A cells transfected with antimiR-93 and a significant decrease in caspase-3/7 activity in premiR-93-transfected MCF-10A cells compared with respective control cells (Figure 5A).

Estrogen is known to induce DNA damage and increased DNA damage is suggested to be a prerequisite step for the initiation and development of estrogen-induced breast cancer (4,5,27). Therefore, to determine the effect of miR-93 on cellular DNA damage, we quantified DNA 8-OHdG levels as a marker of DNA damage in MCF-10A cells transfected with either antimiR-93 or premiR-93. About 2.5-fold increase (P <0.05) in 8-OHdG levels was observed in E2-treated MCF-10A cells compared with vehicle-treated control MCF-10A cells (Figure 5B). AntimiR-93 transfection significantly decreased 8-OHdG levels in MCF-10A cells compared with respective negative control, whereas premiR-93 transfection significantly increased DNA damage compared with premiR negative control-transfected MCF-10A cells (Figure 5B).

Discussion

miRNAs are involved in the regulation of numerous cellular processes, including metabolism, development, proliferation, differentiation and apoptosis (15,16). In this study, we show that miR-93 is a post-transcriptional repressor for *NRF2*. The NRF2 antioxidant pathway is an important cellular defense mechanism against oxidative stress, genotoxicity and tumor formation (5,22,28,29). Estrogen treatment inhibits NRF2 expression during breast carcinogenesis but the mechanisms that regulate NRF2 expression, however, remain poorly understood (5). The miRanda prediction algorithm identified one conserved miR-93 targeting site within the *NRF2* mRNA 3′-untranslated regions of both humans and rats. We have stepwise examined this prediction and found that miR-93 is involved in the regulation of *NRF2* expression at post-transcriptional level.

The expression and oncogenic role of miR-93 in different cancer types have been suggested (21,30–33). In breast cancer, overexpression of miR-93 has been correlated with proliferation and tumor progression (34,35). In estrogen-induced breast carcinogenesis, estrogen-metabolism-mediated oxidative stress is suggested to play an important role (1,2,7,12,13). NRF2 is an antioxidant-responsive transcription factor that is suggested to play an important role in antioxidant-mediated prevention of oxidative stress (5,22,23,29). Expression of a miRNA is inversely correlated to the protein expression of its target genes (20,21,34) but the connection between *NRF2* and miR-93 is not well elucidated. We observed a significant upregulation of miR-93 and significant downregulation of NRF2 in E2-treated rat mammary and mammary tumor tissues and in E2-treated human breast epithelial cells, MCF-10A and human breast cancer cells T47D, compared with their respective controls (Figures 1 and 2). Conversely, antioxidant vit C treatment provided a chemopreventive effect by decreasing miR-93 and increasing NRF2 expression (Figures 1 and 2). Our laboratory has previously established the protective role of vit C against E2-induced breast carcinogenesis (2). In this study, we identified that E2 treatment decreased NRF2 levels through induction of miR-93 expression (Figures 1 and 2; Table I) and protective effects of antioxidant vit C

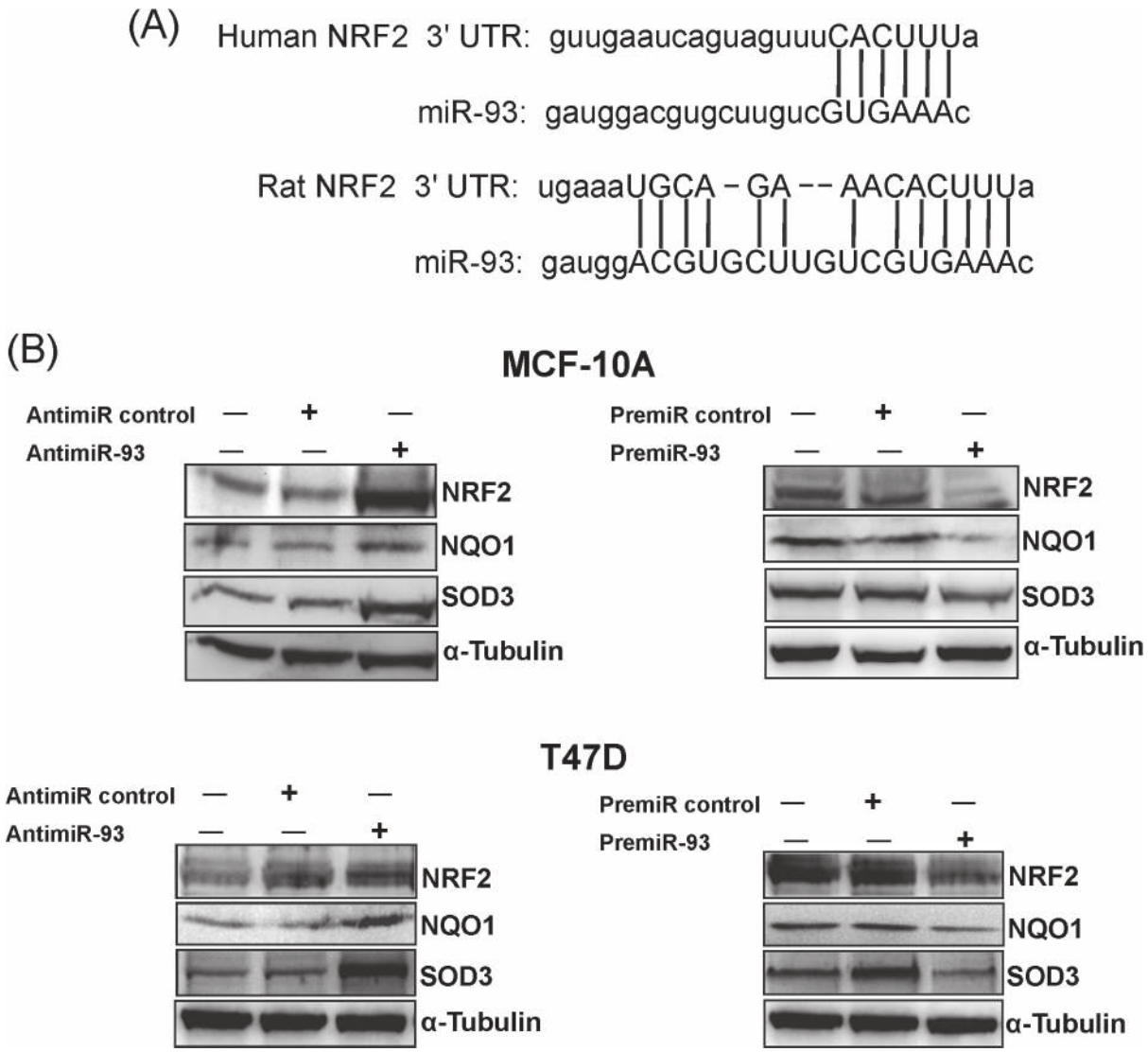

Fig. 3. NRF2 is a potential target for miR-93. (**A**) Schematic of human and rat *NRF2* mRNA 3′-untranslated regions containing potential miR-93 binding site. (**B**) Representative western blots showing effects of silencing or exogenous expression of miR-93 after transfection with antimiR-93 or premiR-93, respectively, on expression of NRF2 and NRF2-regulated antioxidant proteins NAD(P)H:quinone oxidoreductase and superoxide dismutase 3.

treatment might be mediated through inhibition of miR-93 expression and thus through corresponding increase in NRF2 expression (Figures 1 and 2; Table I). MiR-93 is one of the member of miR-106b-93-25 cluster, which is located in the 13th intron of the protein coding gene *minichromosome maintenance complex component 7* (36). Upstream region of miR-106b-93-25 cluster contains a conserved core binding sequence for the forkhead transcription factors (FOXOs) (37). Forkhead transcription factors are redox-sensitive transcription factors and downregulation of these transcription factors by vit C has been shown (38). Thus, vit C-mediated inhibition of oxidative stress and downregulation of forkhead transcription factor proteins might be a possible mechanism involved in the downregulation of miR-93. However, it warrants further investigation. By suppression or ectopic expression of miR-93 using premiR- and antimiR-transfection approaches, we have established that NRF2 is a potential target for miR-93 (Figure 3). Li *et al.* (39) have recently shown inverse correlation between miR-93 expression and its suggested target gene *NRF2* in rat liver tissues. However, in their target validation studies using HEK 293 cell line that has very low levels of endogenous NRF2 expression, they could not find such relationship between miR-93 and NRF2 (39). To the contrary, our target validation studies using MCF-10A and T47D breast epithelial cell lines validate NRF2 as a target of miR-93. Significant downregulation of NRF2 in breast cancer cases and upregulation of it in human colon, pancreatic and liver cancer cases indicate organ- and cell-specific nature of its expression (40–42). The use of different validation models (human breast epithelial cell lines) in this study demonstrates miR-93–NRF2 interaction. Downregulation of NRF2 has been found in breast cancers (5,22) and we have earlier demonstrated that treatment with antioxidants inhibit E2-induced breast carcinogenesis through NRF2-mediated increased expression of antioxidants (5).

Having established that E2 or vit C treatment can manipulate miR-93 expression and NRF2 pathway, we next examined what functional consequences miR-93 would have on tumorigenic properties of breast epithelial cell line, such as colony and mammosphere formation, and apoptosis. We found that suppression of miR-93 and corresponding increased NRF2 expression, decreased colony formation, mammosphere formation, cell migration, apoptosis and DNA damage in MCF-10A cells. Overexpression of miR-93 and corresponding decreased NRF2 expression, increased oncogenic potential of cells, as evidenced by increased colony and mammosphere formation, cell migration, apoptosis and DNA damage (Figures 4 and 5). The mechanism by which miR-93 increases oncogenic potential of cells is most likely through inhibition of NRF2 as redox status

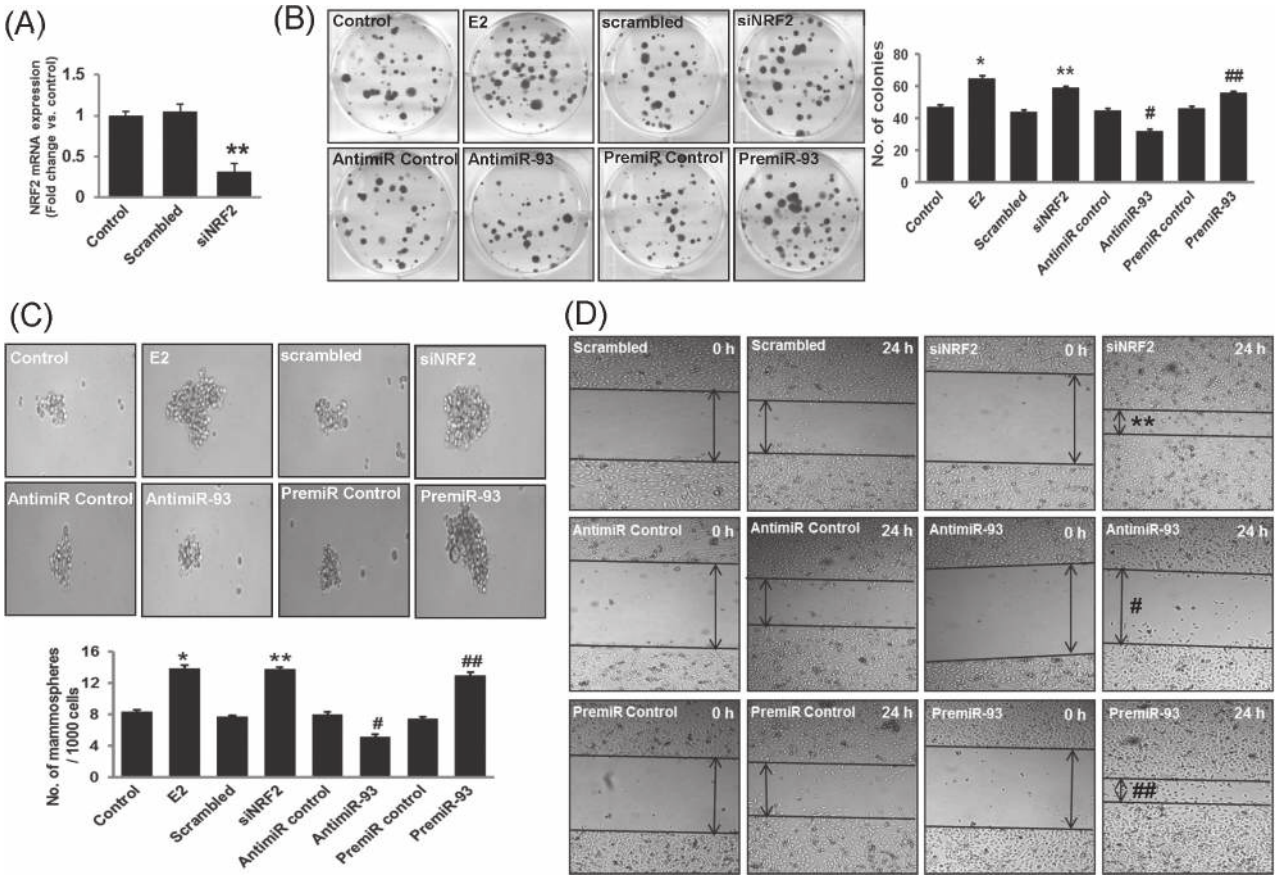

Fig. 4. Overexpression of miR-93 increases and silencing of miR-93 decreases clonability, mammosphere formation and migratory properties of MCF-10A cells. PremiR-93, antimiR-93, siNRF2, premiR negative control and antimiR negative control or scrambled siRNA were transfected in MCF-10A cells for 48 h. Transfected MCF-10A cells were used for colony formation, mammosphere formation and migration assays. (**A**) Inhibition of NRF2 mRNA expression in siNRF2-transfected MCF-10A cells. (**B**) Five hundred parental MCF-10A and transfected MCF-10A cells were seeded in triplicate in six-well plates. E2 (10 nM, 72 h)-treated parental cells were used as a positive control for the assay. After 8 days of incubation period, colonies were photographed and counted. The bar graph is derived from the colonies counted and represents a mean ± standard error of the mean of three assays. (**C**) Parental MCF-10A cells and transfected MCF-10A cells were seeded in suspension in ultra-low attachment plates. E2 (10 nM)-treated parental cells were used as a positive control for the assay. After 8 days of incubation, mammospheres formed in suspension were photographed and counted and representative photomicrographs from triplicates are presented. The bar graph is derived from counted mammospheres and represents a mean ± standard error of the mean of assays in triplicate. (**D**) Photomicrographs demonstrate the results of the *in vitro* migration of transfected MCF-10A cells using the simple scratch technique. Cells were grown in monolayer, scratched and incubated for 24 h. '*' and '**' indicate P value <0.05 compared with vehicle-treated MCF-10A cells and scrambled-transfected MCF-10A cells, respectively. '#' and '##' indicate P value <0.05 compared with antimiR-negative control-transfected MCF-10A cells and premiR negative control-transfected MCF-10A cells, respectively.

of the system plays an important role in regulation of proliferation of cells including stem cells (43,44). NRF2 is known as the central component of a redox control mechanism (5,22,45) and dampening it by miR-93 may cripple the overall redox regulation by impacting the expression of many reactive oxygen species (ROS) detoxifying genes, whose expression is dependent on NRF2 presence (5,22,45). ROS are emerging as critical regulators of stemness and pluripotency of stem cells (46–48). Hochmuth *et al.* (43) have recently demonstrated the regulatory role of NRF2 in stem cells proliferation. They demonstrated that the repression of NRF2 in intestinal stem cells is required for intestinal stem cells proliferation. They further suggested that NRF2 exerts this function in intestinal stem cells by regulating the intracellular redox balance (43). Similarly, in this study, increased colony and mammosphere formation as well as cell migration in NRF2-silenced cells suggests an important role of NRF2 in regulation of stem cell proliferation (Figure 4). Involvement of modulation of the redox balance in response to treatment with ROS-inducing compound like paraquat as well as mutants for ROS detoxifying enzyme catalase in proliferation of the cells has also been shown (49,50). Moreover, treatment with antioxidants *N*-acetyl-cysteine and glutathione has been shown to be sufficient to limit the cells' proliferation potential (51). NRF2 has been shown to be involved in inhibition of cellular plasticity and motility as well as in inhibition of cell growth by inducing cell cycle arrest (52,53). Therefore, it is logical to suggest that oncogenic potential of miR-93 is due to inhibition of NRF2 in mammary tissues during breast carcinogenesis.

Previously, miR-93 has been shown to be a direct regulator of several tumor suppressor genes like *FUS1*, *PTEN*, *ZBTB4* and *p21* (20,21,34). It is quite possible that miR-93 might be regulating these genes as well in our system and needs to be explored in future studies. In this study, we concentrated on the role of miR-93 in *NRF2* regulation. Our results with *NRF2*-knocked down cells mimicked the results of miR-93 over-expression (Figure 4). We have earlier reported that inhibition of NRF2 is involved in increased oxidative DNA damage *in vivo* and *in vitro* (5). In this study, increased colony and mammosphere formation, and cell migration in *NRF2*-knocked down MCF-10A cells further confirms antioncogenic potential of NRF2 (Figure 4). Moreover, we have earlier shown that E2-metabolism-mediated oxidative stress plays an important role in the development of breast cancer (1,2,7) and antioxidants inhibit E2-induced breast carcinogenesis through induction of NRF2 (5). As our findings suggest an important role of NRF2 in the inhibition of carcinogenic potential of the cells, and NRF2 is a potential target of miR-93, it

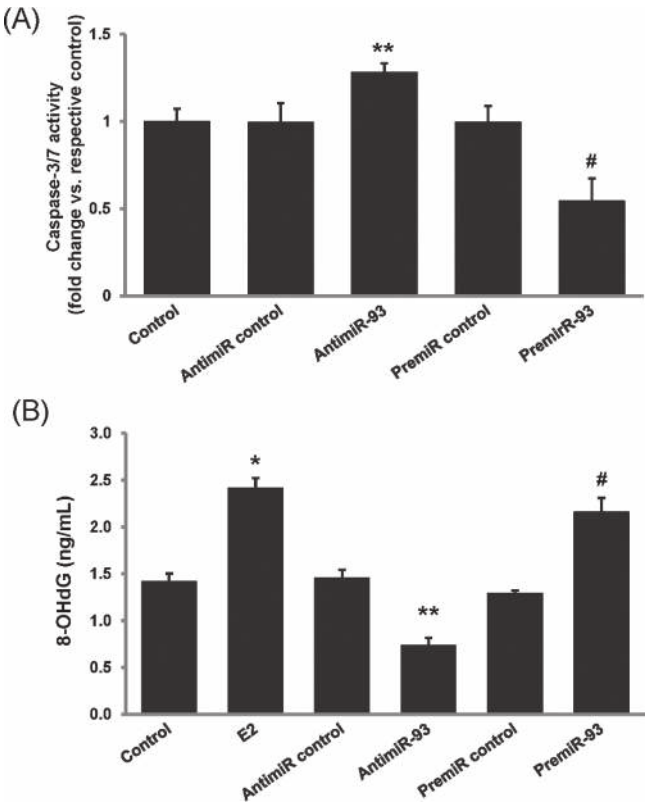

Fig. 5. MiR-93 inhibits apoptosis and increases oxidative DNA damage. PremiR-93, antimiR-93, premiR negative control and antimiR negative control, respectively, were transfected in MCF-10A cells for 48 h. Transfected MCF-10A cells were used for caspase-3/7 activity and DNA 8-OHdG assays. (**A**) Cultured MCF-10A cells were assayed for caspase-3/7 activity as described in the Materials and methods section and the data are reported as an average of fold change (mean ± standard error of the mean) from at least five different experiments compared with respective controls. (**B**) 8-OHdG levels were quantified in parental MCF-10A cells in presence or absence of E2 (50 nM) and transfected MCF-10A cells as described in the Materials and methods section and are presented here as bar graph. These data are presented as an average of values obtained for at least five different samples ± standard error of the mean. '*' indicates P value <0.05 compared with parental vehicle-treated MCF-10A cells. '**' and '#' indicate P value <0.05 compared with antimiR control-transfected MCF-10A cells and premiR control-transfected MCF-10A cells, respectively.

became imperative to find a link between the carcinogenic potential of miR-93 and NRF2. To the best of our knowledge, this study is the first report showing direct regulation of NRF2 by miR-93.

In summary, we have provided several pieces of evidence that suggest that miR-93 exerts its oncogenic potential by downregulating protective antioxidant regulating gene *NRF2* by targeting specific sites in its 3′-untranslated regions in breast cancers. Finally, we show that antioxidants, for example, vit C can inhibit breast cancer cell growth, in part, through inhibition of miR-93, and thus allowing NRF2-dependent antioxidant pathway to function properly. The identification of antioxidants like vit C as inhibitors of miR-93 expression might have significant therapeutic potential and may open a new avenue of research for inhibition of breast cancer.

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