Original Article
Targeting PDK4 inhibits breast cancer metabolism

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Abstract: Dysregulated metabolism in the form of aerobic glycolysis occurs in many cancers including breast carcinoma. Here, we report PDK4 (pyruvate dehydrogenase kinase 4) as key enzyme implicated in the control of glucose metabolism and mitochondrial respiration is relatively highly expressed in breast cancers, and its expression correlates with poor patient outcomes. Silencing of PDK4 and ectopic expression of miR-211 attenuates PDK4 expression in breast cancer cells. Interestingly, low miR-211 expression is significantly associated with shorter overall survival and reveals an inverse correlation between expression of miR-211 and PDK4. We have found that depletion of PDK4 by miR-211 shows an oxidative phosphorylation-dominant phenotype consisting of the reduction of glucose with increased expression of PDH and key enzymes of the TCA cycle. miR-211 expression causes alteration of mitochondrial membrane potential and induces mitochondrial apoptosis as observed via IPAD assay. Further, by inhibiting PDK4 expression, miR-211 promotes a phenotype shift towards a pro-glycolytic state evidenced by decreased extracellular acidification rate (ECAR); increased oxygen consumption rate (OCR); and increased spare respiratory capacity in breast cancer cell lines. Taken together this data establishes a molecular connection between PDK4 and miR-211 and suggests that targeting miR-211 to inhibit PDK4 could represent a novel therapeutic strategy in breast cancers.

Keywords: PDK4, miR-211, altered metabolism, apoptosis, Warburg effect

Introduction

Breast cancer is the most common solid tumor in women with an estimated 2.4 million cases in 2015 [1]. It is also the second leading cause of cancer death in women. Breast cancer is a heterogeneous disease classified by expression or absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [2]. The standard treatment regimen for breast cancer includes chemotherapy, radiation, surgical resection and hormone therapy directed at the receptors outlined above. Existing therapies still fall short in adequately treating breast cancer, especially for triple-negative breast cancers (TNBC) that lack ER, PR, and HER2 expression. Thus, identifying novel therapeutic strategies for breast cancer is necessary to improve patient outcomes.

Tumor cells undergo metabolic reprogramming to utilize glycolysis even in the presence of abundant oxygen while shifting energy derivation away from mitochondrial oxidative phosphorylation (OXPHOS). This metabolic phenomenon is referred to as the "Warburg effect", or aerobic glycolysis. This anomaly is now widely accepted as one of the hallmarks of aggressive cancers, and understanding the mechanistic steps of this process might be beneficial in identifying potential targets for novel breast cancer therapies [3, 4]. Pyruvate dehydrogenase kinase isoform 4 (PDK4) is a member of PDK family located in the mitochondrial matrix of cells. PDK4 inhibits the entry of pyruvate into the TCA cycle by inhibiting pyruvate dehydrogenase activity, thereby switching energy derivation to cytoplasmic glycolysis in lieu of mitochondrial OXPHOS, consistent with the aforementioned Warburg effect. PDK isoforms are highly

upregulated in various other cancers including glioblastoma, lung carcinoma, pancreatic cancer, amongst others [5-8].

Attention has recently been focused on the role of small, non-coding RNA molecules in cancer development known as microRNAs (miRNAs) [9-11] and in breast cancer [12]. miRNAs are a class of short (~22) nucleotide non-coding RNA molecules that bind to the 3' untranslated region (3'UTR) of target messenger RNA to suppress gene expression [13, 14] involved in the modulation of diverse biological processes [15-17]. As a tumor suppressor, miR-211 regulates several biological processes, and its downregulation is associated with increased tumorigenicity in many cancers [18, 19]. However, the regulatory mechanisms underlying miR-211 in breast cancer and in glucose metabolism is not well reported.

In this study, we show that PDK4 is highly expressed in all molecular subtypes of breast cancer. MIR-211 expression is more highly expressed in normal breast tissue compared to breast cancer tissue as seen with *in situ* hybridization experiments. Overexpression of miR-211 supresses the Warburg effect in breast cancer cell lines, resulting in increased pyruvate entry to OXPHOS as shown in metabolic studies conducted using the Seahorse Bioanalyzer. Further, miR-211 overexpression regulates the proliferative and apoptotic behavior of MDA-MB-468 and BT-474 cells. Collectively, we demonstrate that PDK4 is a potential target of miR-211 and our results establish miR-211 as a robust inhibitor of the Warburg effect and promising potential therapeutic target for breast cancer treatment.

Materials and methods

Cell culture

Two breast cancer cell lines, BT-474 and MDA-MB-468, were obtained from the American Tissue Cell Culture (ATCC) and were cultured in RPMI and DMEM high-glucose media containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, in a 5% CO_2 humidified incubator at 37°C.

Plasmids, chemicals and antibodies

A PLKO-mcherry-luc-puro-miR-211 construct and empty vector were purchased from Addgene (Cambridge, MA) and transfected as

described previously [20]. The siRNA construct used to silence PDK4 was purchased from Santa Cruz (sc-39030). The miRCURY LNA™ microRNA ISH Optimization Kit (FFPE) and miR-211 probe was obtained from Exiqon (Woburn, MA). A human breast cancer tissue microarray (TMAH-BRC-03) was obtained from Ray Biotech. The JC-1 kit was obtained from Cayman Chemicals (Ann Arbor, MI). *In situ* cell death detection kit and Fluorescein kits were purchased from Sigma (11684795910). Proteome Profiler Human Apoptosis Array kits (ARY009) and the human pluripotent stem cell antibody array (ARY010) were purchased from R&D Biosystems. Seahorse XFp cell energy phenotype test kits (103275-100), Mito Stress test kits (103010-100) and Glycolysis Stress kits (103346-100) were purchased from Agilent Technologies. Protein concentration was estimated using the bicinchonic acid (BCA) assay (Pierce, Rockford, IL). All antibodies used in this study were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA). The primer sequences were synthesized by IDT and the primer list was provided as listed in Table S1.

TCGA cohort analysis

We collected mRNA expression profiles from The Cancer Genome Atlas (TCGA) invasive breast carcinoma cohort and miRNA cohort using the TCGA data portal (http://cancergenome.nih.gov/). To gather information on ER, PR, and HER2 status, we also downloaded clinical information from the TCGA data portal.

Quantitative real-time PCR

Total RNA was isolated from BT-474 and MDA-MB-468 cell lysates using the Trizol method (Life Technologies). Subsequent quantification was performed using a CFX-96 analyzer. SYBR green primers used in this study were provided as outlined in Table S1. GAPDH was used as the endogenous control. A total RNA concentration of 2 ng/µl was reverse-transcribed to cDNA using miR-211 primers from miRCURY LNA™ miRNA polymerase chain reaction system (Exiqon, Vedbaek, Denmark).

Western blot analysis

Proteins extracted from BT-474 and MDA-MB-468 cells obtained from both control and respective treatments were assayed with multiple antibodies using the immunoblot approach

as described previously [5]. GAPDH was used to confirm equal loading.

Apoptosis protein arrays and stem cell antibody arrays

An apoptosis array (ARY009) detecting the expression level of 35 apoptotic proteins and a human pluripotent stem cell antibody array (ARY010) detecting the relative expression of 15 pluripotent stem cell markers were purchased from R&D Systems (Minneapolis, MN). Approximately 200 µg of total-cell lysates were taken for these arrays and were performed in accordance with the manufacturer's instructions. Signal intensity of each spot was quantified with Image J (Bethesda, MD) and averaged for each specific protein.

IR treatment

For combination treatment with miR-211, an IR dose of 8 Gy was given 48 h after plasmid transfection in both BT-474 and MDA-MB-468 cells using the RS 2000 Biological Irradiator X-ray unit (Rad Source Technologies Inc., Boca Raton, FL, USA). Irradiated cells were further incubated for 24 h before harvesting for Western blot analysis.

Mitochondrial dysfunction and TUNEL assay

Mitochondrial membrane potential was detected using JC-1 fluorescent dye (Cayman Chemicals; Ann Arbor, MI) as described previously (21). Both the EV- and miR-211-treated BT474 and MDA-MB-468 cells were washed once with PBS and subjected to JC-1 staining for 10 min at 37°C. Cells were washed again with PBS and imaged for the JC-1 monomer and aggregate with fluorescence microscopy. The shift from red to green fluorescence indicates the collapse of mitochondrial membrane potential. To measure cell death, the TUNEL assay (Roche; Indiana polis, IN) was performed in both BT-474 and MDA-MB-468 cells treated with miR-211 following the manufacturer's instructions.

Metabolic analysis

BT-474 and MDA-MB-468 cells were plated at a density of 20,000/well in an XFp 8-well plate. Two days before, cells were transfected with miR-211. On third day, cells were transferred to an 8-well moat chamber and allowed to grow overnight with media exchange occurring 1 hour before the assay with XFp media to determine the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). For the cell energy phenotype assay, FCCP and oligomycin were diluted into XFp media and loaded into an accompanying cartridge to achieve final concentrations of 10 µM; for the mitostress assay, oligomycin, FCCP and rotenone/antimycin A were diluted to achieve final concentrations of 1.0 µM and 0.5 µM, respectively. Injection of drugs into the medium occurred at the time points specified. These experiments were done to define a basal OCR, ATP-linked OCR, proton leak, maximal respiratory capacity, reserve respiratory capacity, and non-mitochondrial oxygen consumption. All reagents were purchased from Seahorse Biosciences.

Immuno-paired antibody detection (IPAD) analysis

The IPAD assay (ActivSignal, LLC, Natick, MA) was used to study the expression and phosphorylation status of 70 different human protein targets covering 20 major signaling pathways. In this assay, we used paired antibodies for each target protein and detection was contingent upon whether both antibodies in a pair bound to a specific target protein. Detection of the paired antibodies was facilitated by special DNA barcodes conjugated to antibodies, which were quantified using Next Generation Sequencing or Fluidigm digital PCR.

In situ hybridization of miR-211

Breast cancer tissue microarrays (TMA) were used in this experiment. The TMA was de-paraffinized and digested with proteinase-K for 10 min. Next, the TMA was hybridized with a DIG-labeled mercury LNA miR-211 probe (Exiqon, MA, USA) for one hour at 55°C. An alkaline phosphatase-conjugated secondary antibody and an anti-DIG antibody were used to detect the digoxigenins as per the manufacturer's instructions. Raw images were captured using the Olympus BX61 fluorescent microscope.

Immunohistochemistry

For immunohistochemical analysis, we used breast cancer TMA (TMAH-BRC-03). The TMAs
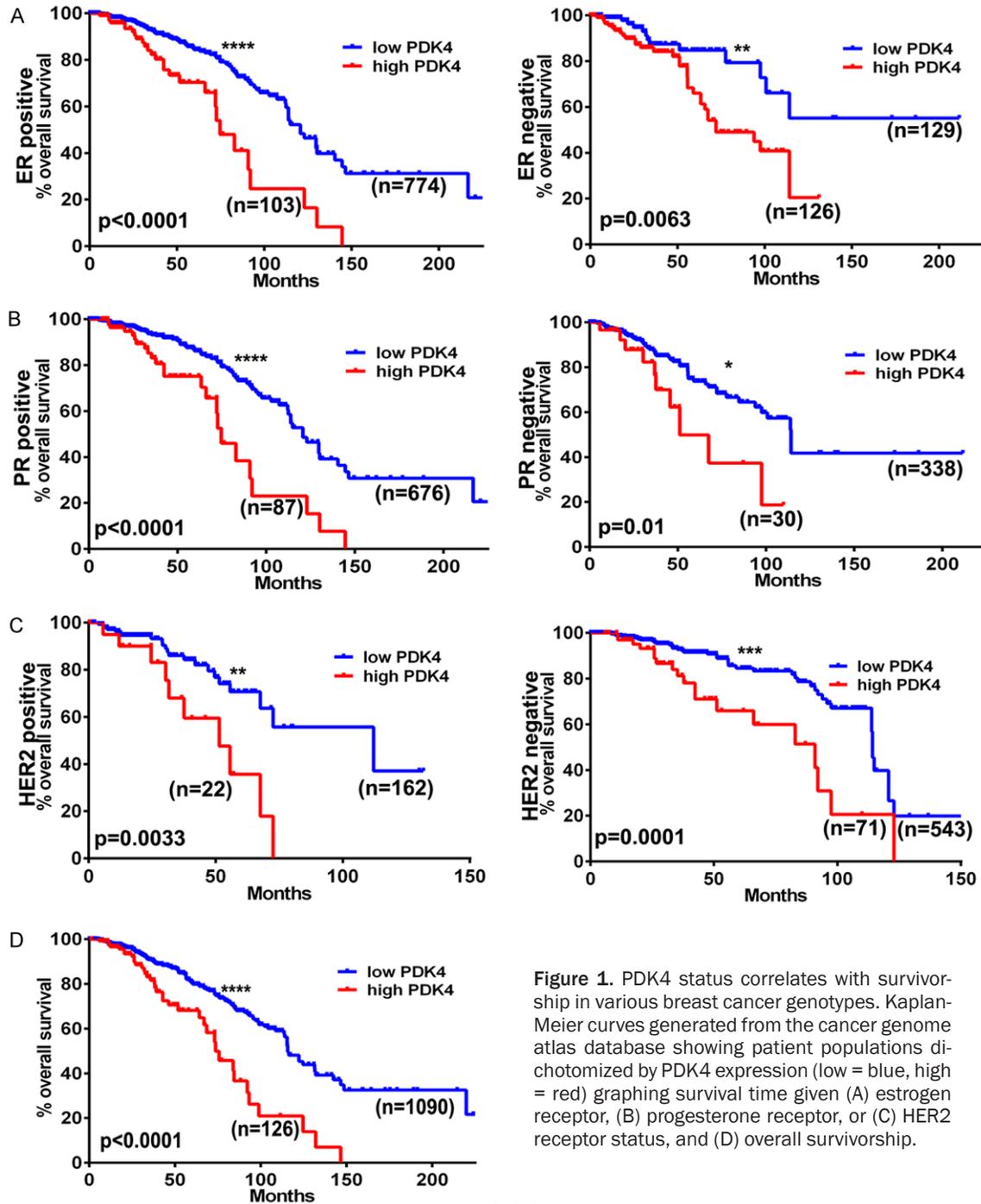
Figure 1. PDK4 status correlates with survivorship in various breast cancer genotypes. Kaplan-Meier curves generated from the cancer genome atlas database showing patient populations dichotomized by PDK4 expression (low = blue, high = red) graphing survival time given (A) estrogen receptor, (B) progesterone receptor, or (C) HER2 receptor status, and (D) overall survivorship.

were de-paraffinized in xylene and rehydrated in graded ethanol solutions. Antigen retrieval was carried out with 10 mM of citrate buffer (pH 6.0) at boiling temperature for 60 min and permeabilization was carried out in in 0.1% Triton-X-100. DAB staining was done to evaluate the expression of PDK4.

Statistical analysis

The results shown are represented as mean ± SD. Graph pad 5.0 was used to perform student's *t*-tests to evaluate the differences between the control and treated groups. For multiple comparisons within TCGA and GOBO
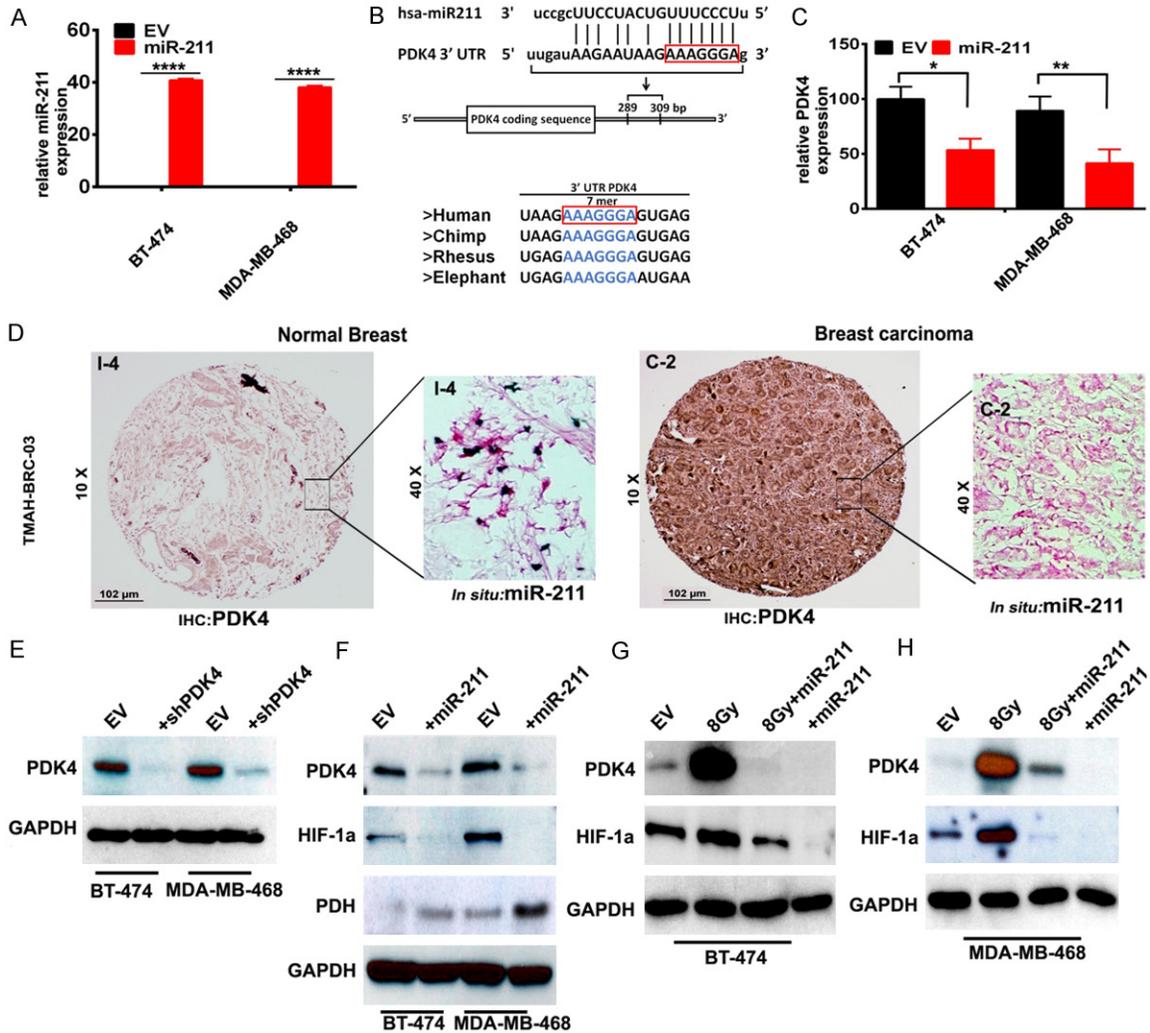
Figure 2. miR-211 is down regulated in breast cancer. (A) RT-PCR analysis to detect miR-211 endogenous levels in BT-474 and MDA-MB-468 cell lines. (B) Schematic representation of miR-211 sequence alignment with 3'UTR of human PDK4. The nucleotides in red box are putative positions of interaction. The region of miR-211 binding to PDK4 is conserved across the species. (C) RT-PCR analysis to detect mRNA levels of PDK4 in empty vector and miR-211 over expressing plasmid transfected BT-474 and MDA-MB-468 cells. (D) Negative IHC of normal breast tissue for PDK4 protein alongside inset *in situ* hybridization showing expression of miR-211 RNA (left panel); IHC of breast carcinoma demonstrating PDK4 positivity and inset image showing relative absence of miR-211 RNA via *in situ* hybridization (right panel). (E) Western blot analysis of PDK4 in PDK4 silenced (shPDK4) cells (F) miR-211 transfected cells probed for PDK4, HIF1α and PDH proteins using specific antibodies (EV = Empty vector). Total protein lysates obtained after 8 Gy radiation, miR-211 transfection, and 8 Gy radiation + miR-211 transfection from (G) BT-474 and (H) MDA-MB-468 cells were collected after 72 h. Immuno blot analysis of PDK4 and HIF1α levels were shown in Panel (G and H) respectively. GAPDH was used as a loading control.

datasets, ANOVA tests were used. All *p*-values were considered statistically significant with a value <0.01; 0.005.

Results

PDK4 expression is upregulated in breast cancer patients

To study the clinical relevance of PDK4 expression in breast cancer and to define its possible prognostic value, we performed datamining studies using TGCA data. It is well established that clinical outcomes in breast cancer are dependent in part on molecular subtype (i.e., ER, PR, and HER2 status) [22]. Therefore, we plotted Kaplan-Meier survival curves from 1216 cases using mRNA and clinical data to define the significance of PDK4 on survival within previously defined subtypes. In **Figure 1A**, we plotted survival curves for the speci-

mens with ER positivity (n = 887; P<0.0001) and ER negativity (n = 255; P = 0.0063). **Figure 1B** shows data for PR positive (n = 763; P< 0.0001) and PR negative (n = 368; P = 0.01) cases; **Figure 1C** shows the plot for HER2-positive (n = 184; P = 0.0033) and HER2-negative (n = 614; P = 0.0001) cases. **Figure 1D** shows the data plotted from all 1216 patients (P<0.0001). Overall, high expression of PDK4 was significantly associated with poor survival in each of the subtypes. A strong correlation was observed in the all-case curves shown in **Figure 1D**. Together, these results suggest that expression of PDK4 has potential as a prognostic marker and therapeutic target in breast cancer.

PDK4 is a direct target of miR-211

To further our understanding on the use of miR-NAs and to identify the potential targets in aerobic glycolysis, we reviewed available literature and public databases including TargetScan and miRanda and concluded that PDK4 is a potential target of miR-211 [23]. To study levels of endogenous miR-211 and levels after overexpression, we transfected BT-474 (ER$^+$, PR$^+$ and HER2$^+$) and MDA-MB-468 (ER$^-$, PR$^-$ and HER2$^-$) [24] cells with a miR-211 overexpression plasmid and an empty vector (EV). RT-PCR studies showed a significant increase in miR-211 levels when compared to the empty vector. No miR-211 expression was seen in EV-transfected cells (**Figure 2A**). Next, we confirmed the presence of a seed sequence of the predicted miR-211 target binding site within the PDK4 mRNA sequence located at the 302-308 position in the 3'UTR region, which is highly conserved across four species [42]. This data was used to demonstrate PDK4 as a putative target of miR-211 (**Figure 2B**). To determine whether miR-211 influences PDK4 expression, we transfected BT-474 and MDA-MB-468 cells with the miR-211 plasmid. We observed significant silencing of PDK4 mRNA levels upon miR-211 transfected cells when compared to the empty vector transfected cells (**Figure 2C**). Because increased PDK4 levels have been associated with increased metabolic rate within tumors [25], we checked the existence of a similar correlation using a human breast cancer tissue microarray (TMAH-BRC-03, Ray Biotech) comprised of 39 metastatic and 38 benign breast tumor tissues using immunohistochemistry.

DAB-stained tissues showed intensive staining of PDK4 in breast carcinoma tissues (C-2) when compared to normal breast tissues (I-4). To test the expression of miR-211 in clinical breast cancer specimens, we performed *in situ* hybridization assay using the human breast cancer tissue microarray (TMA). Tumor specimens showed increased positivity for PDK4 expression when compared to the normal breast tissue. Likewise, spots from normal breast tissue demonstrated increased expression of miR-211 when compared to the tumor counter parts (**Figures 2D** and S1A). To corroborate our TMA and *in situ* findings we plotted the Kaplan-Meier survival curve for miR-211 using previously described TCGA dataset in **Figure 1**. High miR-211 expression promotes increased survival when compared to its low expression (Figures S1B and S1C). These results show that both PDK4 and miR-211 levels are inversely correlated in breast cancer cells, humanderived tissues and in the TCGA datasets.

We next conducted a transient transfection of shPDK4 plasmid and observed decreased levels of PDK4 as demonstrated by Western blot (**Figure 2E**). We conducted this experiment to compare the levels of PDK4 in both shPDK4 and miR-211 treatments. Since we observed negligible amounts of endogenous miR-211 (**Figure 2A**), we overexpressed miR-211 using transient transfection. Transfected miR-211 cells showed significantly decreased expression levels of both PDK4, HIF1α and the results obtained are comparable shPDK4 treatments. Interestingly, the levels of PDH are observed to be highly expressed in the miR-211 transfected cells (**Figure 2F**).

Recent reports suggesting the decline of tumor recurrence with radiation is encouraging but is not consistent from patient to patient [26]. Our laboratory previously showed that miR-211 overexpression treatment caused DNA fragmentation in glioma stem cells even at sublethal (i.e., 5 GY) doses of ionizing radiation (IR) [20]. Thus, as a proof of concept, we examined the effects of miR-211 transfection on BT-474 and MDA-MB-468 cells exposed to 8 Gy IR. After 72 h, we harvested the samples and checked expression levels of PDK4 and HIF1α. IR treatment increased PDK4 and HIF1α levels, whereas miR-211 treatment reduced levels of these proteins. Interestingly, the combination
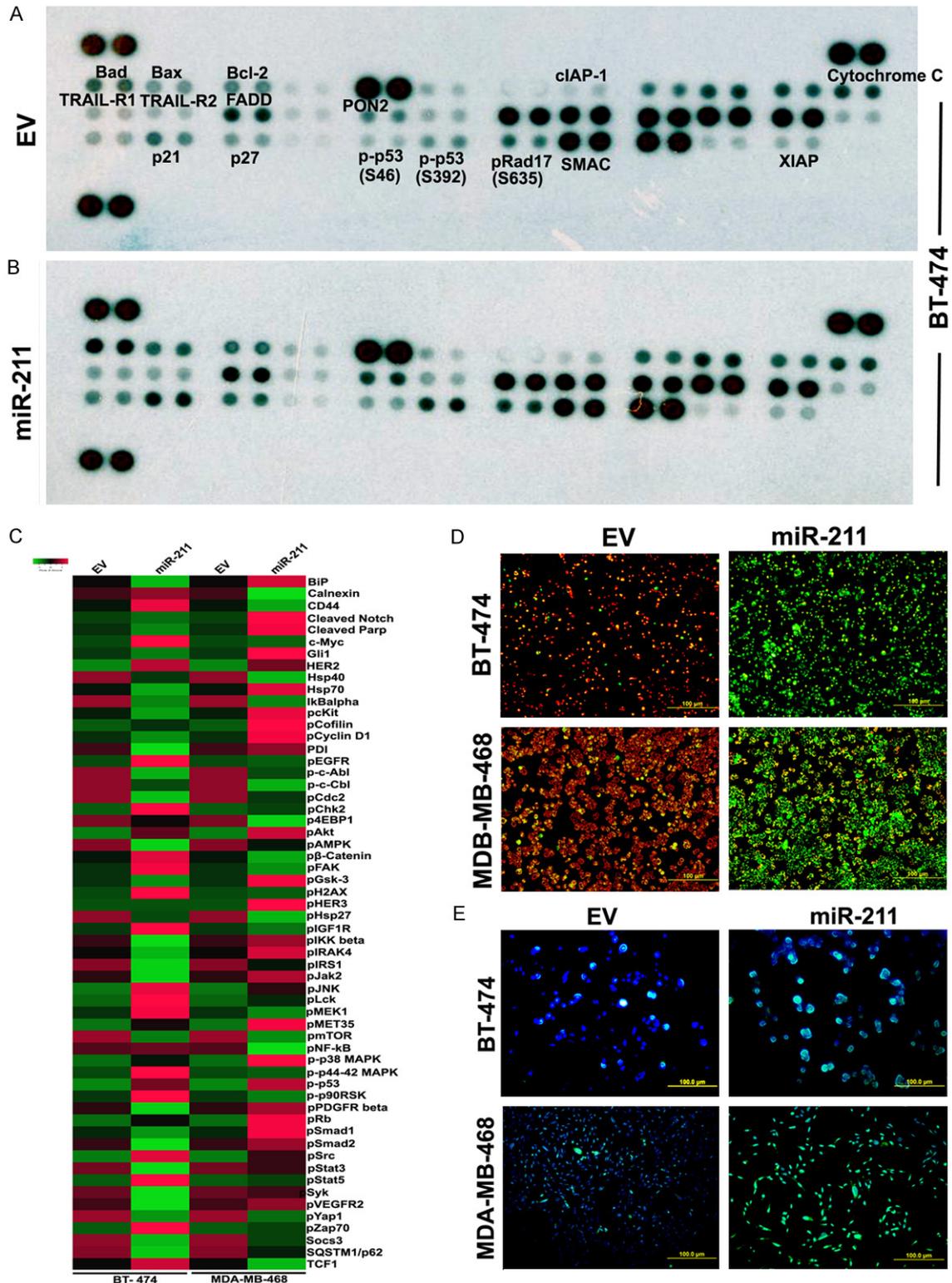
Figure 3. miR-211 promote mitochondrial apoptosis of breast cancer cells *in vitro*. Representative expression levels of various apoptosis -related proteins in BT-474 cells transfected with EV (A and B) miR-211. Illustrated images showed increased levels of several apoptotic proteins after mir-211 silencing. (C) BT-474 and (D) MDA-MB-468 cells were transfected with EV and miR-211 were subjected to IPAD assay, which measure simultaneous expression or activation of 70 proteins involving various activated signaling pathways in tumors. The differential expression is presented as heatmaps using a free software available online (www.shinyheatmap.com). (D) Both BT-474 and MBA-MD-468 cells transfected with miR-211 and EV were treated with JC-1 dye to record the alteration in mitochondrial membrane potential (Red = live cells; Green = dead cells). (E) Cells were stained for apoptosis using TUNEL assay.
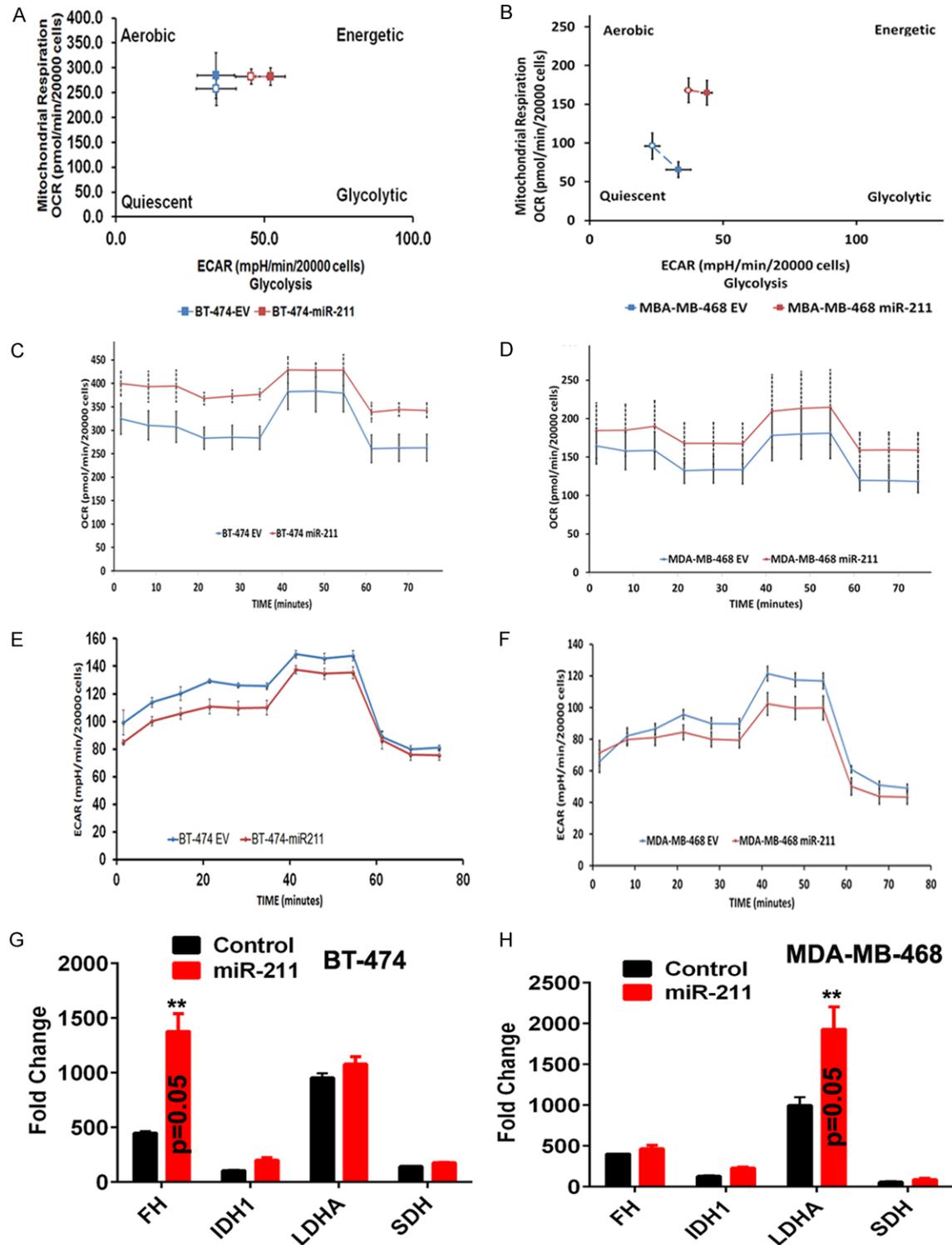
Figure 4. miR-211 suppresses the glycolytic pathway; modulate cellular bioenergetics. Both the EV- and miR-211 transfected cells were plated in Seahorse XFp 8 well plates. For all the experiments that involve Seahorse XFp, cell medium is replaced with bicarbonate free medium, one hour before use. Cell energy phenotype (A and B) assay was performed to measure the metabolic phenotype in EV and miR-211 transfected cells. Mitostress test (C and D) and Glycolytic stress test (E and F) were performed to assess the spare respiratory capacities, oxygen consumption rates (OXPHOS) and extracellular acidification rates (glycolysis) in EV and miR-211 transfected BT-474 and MDA-MB-468 cells. All the experiments were performed in duplicates. (G and H) RT-PCR analysis.

treatment (8 Gy+miR-211 treatment) showed comparable results of miR-211 overexpression. These results confirm PDK4 as a target of miR-211 in *in vitro* breast cancer models (**Figure 2G** and **2H**).

miR-211 overexpression promotes mitochondrial apoptosis in BT-474 cells

Our previous study conducted on the role of miR-211 in glioblastoma demonstrated that miR-211 regulated the tumor growth by inducing apoptosis [20]. In this study, we aimed to delineate the effects of miR-211 on mitochondrial-mediated apoptosis in the BT-474 cell line. For this experiment, BT-474 cells were transfected with either an EV or a miR-211 overexpression plasmid. To verify if miR-211 transfection induced apoptosis, we loaded the human apoptosis array with total protein lysates of EV and miR-211 treated BT-474 cells. A comparison of pictographs of the spots between EV-treated and miR-211-treated arrays showed increased expression of mitochondrial apoptosis-related proteins including Bad, Bax, FADD, SMAC, p21, p27 and p53$_{s46}$, and p53$_{s392}$. In total, 16 out of 35 proteins were upregulated in miR-211-treated cells when compared to the EV treatment (**Figures 3A**, **3B** and S2A). To further study the effects of miR-211 treatments on its downstream signaling, we performed an IPAD assay on BT-474 and MDA-MB-486 cells. The IPAD assay is a relatively new platform that can simultaneously study the activation of approximately 20 major active signaling pathways in cancer. We observed increased phosphorylation of retinoblastoma protein (Rb), p38MAPK, pGSKβ, and p53 in both the cell lines tested, implicating the role of miR-211 as a potential tumor suppressor in cancer pathways (**Figure 3C**). As we observed the induction of mitochondrial apoptosis in miR-211-treated cells (**Figure 3A**, **3B**), we next measured the mitochondrial membrane potential using JC-1 dye. EV-treated BT-474 and MDA-MB-468 cells showed J-aggregates as shown in red fluorescence. The miR-211-treated cells showed green fluorescence, indicating an onset of apoptosis. These results confirm that miR-211 may induce apoptosis through mitochondrial dysfunction (**Figures 3D**, S2B and S2C). To further asses the percent apoptosis, we conducted the Tunel assay and showed increased cell death in miR-211-treated cells compared to EV treatments in both BT-474 and MDA-MB-468 cells (**Figures 3E**, S2D). These results suggest that miR-211

may control breast cancer progression by inducing mitochondrial apoptosis and by influencing the expression of p53, pRb, and p38.

miR-211 reverses the Warburg effect in BT-474 and MDA-MB-468 cells

The data presented in **Figure 2** shows that miR-211 treatment reduces PDK4 and increased the expression of PDH expression in miR-211 transfected cells. It is widely accepted that silencing of PDK4 and overexpression of PDH reverses metabolic reprogramming consistent with the Warburg Effect. To study if miR-211 plays such a role in breast cancer cells, we conducted cell energy phenotype assays using the XFp Seahorse Bioanalyzer system. This assay delineated the metabolic phenotypes of BT-474 and MDA-MB-468 under both baseline and miR-211-overexpressed conditions. BT-474 and MDA-MB-468 cells demonstrated a glycolytic phenotype, while miR-211 treatment induced an energetic phenotype in both lines. These results indicate that miR-211 overexpression reduces the ECAR (extracellular acidification rate) of breast cancer cells and shifts their energy derivation toward OXPHOS (**Figure 4A**, **4B**). Next, we queried alterations of the major respiratory chain complexes in BT-474 and MDA-MB-468. In this experiment, both BT-474 and MDA-MB-468 cells treated with EV and miR-211 overexpression were exposed to the mitochondrial inhibitors rotenone and antimycin A. MiR-211-treated cells increased spare respiratory capacity compared to test controls (**Figure 4C** and **4D**). Further, we extrapolated glycolytic flux by measuring the extracellular acidification rate (ECAR) (i.e., the secretion of protons into the extracellular medium) in BT-474 and MDA-MB-468 cells. Under normal conditions, ECAR profiles were reciprocal of OCR profiles (**Figure 4E**, **4F**), in both cell lines. These results indicate that miR-211 could metabolically control breast cancer by favoring oxidative phosphorylation over glycolysis. We next conducted RT-PCR analysis on enzymes of TCA cycle and observed their increased expression confirming the aforementioned effects of miR-211 on breast cancer cells (**Figure 4G**, **4H**).

miR-211 reduces stemness in breast cancer cells

Earlier reports suggest that overexpression of certain miRNAs in breast cancer cell lines induces stemness [27]. To analyze the possible
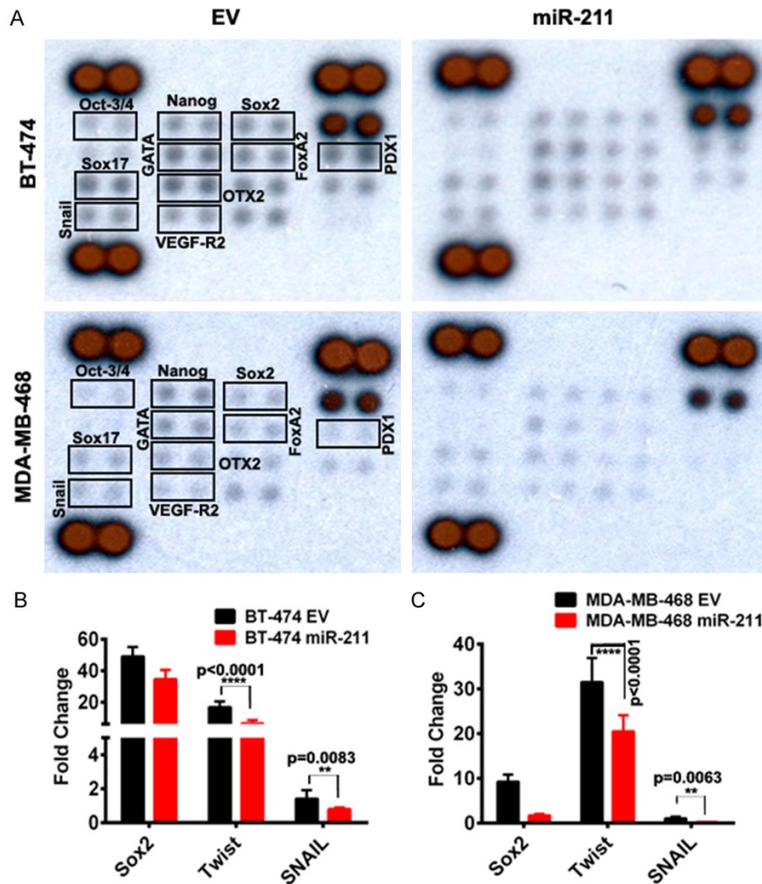
Figure 5. miR-211 overexpression reduced breast cancer stemness *in vitro*. (A) Around ~200 mg of total protein was extracted from EV and miR-211 transfected breast cancer cell. The experiment was conducted in accordance with the manufacturer's instructions. Human pluripotent stem cell antibody array (#ARY010) was used to measure the change in the expression profiles of stemness related markers. Representative pictographs showed decreased levels of stem cell markers in miR-211 treatments. (B and C) Validation of differentially expressed stem cells markers in (A) using RT-PCR analysis.

obtained in this present study emphasize the role of miR-211 overexpression in the breast cancer by induction of mitochondrial apoptosis, stemness; promoting radio sensitivity and reversal of Warburg Effect (**Figure 6**).

Discussion

Micro RNAs that post-transcriptionally modify the expression of millions of genes are an interesting therapeutic target that have the potential to alter cellular processes characteristic of malignancy [28-31]. In this study, we have provided evidence that the glycolytic enzyme PDK4 is overexpressed in breast cancer tissue and drives a functional metabolic shift toward glycolysis. We have shown that PDK4's overexpression in breast cancer patients is correlated with poor prognoses for patients irrespective of their ER, PR, and HER2 status and irrespective of their histological subtype. Furthermore, we have demonstrated that miR-211 can be utilized to alter the expression of HIF1α and PDK4 in BT-474 & MDA-MB-468 breast cancer cell lines in vitro by targeting the 3'UTR of

role of miR-211 in inducing a stem-like phenotype, we overexpressed miR-211 in BT-474 and MDA-MB-468 cells and analyzed various stem cells markers using a stem cell array (**Figure 5A**). Treatment of BT-474 and MDA-MB-468 cells with miR-211 reduced the expression of several transcription factors known to regulate stemness. Among these were Oct3/4, Otx2, and Nanog, which form a transcriptional complex in embryonic stem cells with Sox2. To further investigate the effect of miR-211 on properties of stemness, we checked the real-time PCR analysis of stem-cell markers Sox2, Twist and SNAIL. RT-PCR analysis showed that cells overexpressing miR-211 have reduced expression of these markers compared to EV-treated cells (**Figure 5B**, **5C**). Collectively, the results

PDK4. The application of miR-211's ability to reduce HIF1α and PDK4 expression seems to have particular utility in the context of concurrent ionizing radiation, which we demonstrated causes an increase in PDK4 and HIF1α levels. Even when cells were treated with IR and miR-211 simultaneously, PDK4 and HIF1α levels were reduced.

The Warburg Effect is a shift observed in cancer cells from oxidative phosphorylation to glycolysis that allows for cancers to disproportionately utilize available nutrient stores [32]. A 2016 study by Han et al. showed another miRNA's ability to block the Warburg Effect through post-transcriptional modification of PDK4 in hepatocellular carcinoma [33]. Our evidence
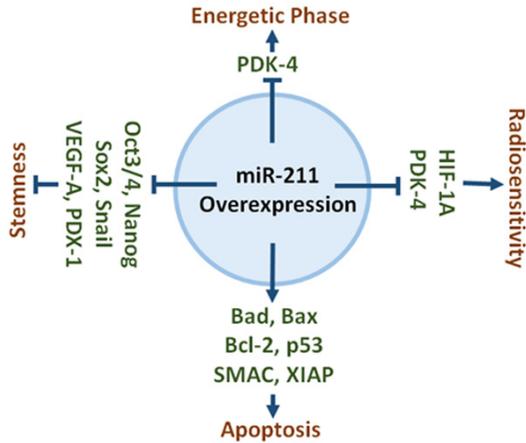
Figure 6. Schematic representation of role of miR-211 in different functions. Overexpression of miR-211 increase the expression of apoptotic genes, reduce the expression of stemness markers, and decrease the expression of metabolic markers.

provided by cell energy phenotype assays run using the Seahorse Bioanalyzer provides quantitative evidence suggests that a similar end result can be accomplished with the use of miR-211 in breast cancer. Initially, both cell lines demonstrated a glycolytic phenotype consistent with the Warburg effect that would be expected in cancer. However, with miR-211 overexpression, a more energetic phenotype as demonstrated by reduced ECAR and increased OCR. This demonstration of miR-211 as a controller of metabolism in breast cancer cell lines can be connected back to its effects on expression levels of PDK4 and HIF1α, as PDK4 and HIF1α are both implicated in mediating glycolysis, while their inhibition shifts metabolism toward an energetic phenotype.

There is additionally evidence that HIF1α and PDK upregulation may contribute to the evasion of apoptosis following radiation therapy in hypoxic malignancies [34, 35]. A 2012 study by Duru et al. demonstrated a HER2-mediated survival in which radioresistance is developed by HER2-positive breast cancer stem cells [36]. Our treatment of the BT-474 & MDA-MB-468 cell lines with 8 Gy radiotherapy seems to suggest that increases in PDK4 & HIF1α protein expression occur regardless of HER2 status, as BT-474 is HER2+ while MDA-MB-468 is HER2-. More importantly, our studies indicate that addition of miR-211 may attenuate the expression of these two proteins in both cell lines,

thereby inhibiting the typical changes that occur following radiotherapy and allow for a pro-apoptotic state. The maintenance of radio-sensitivity during ionizing radiation would make this treatment more efficacious, suggesting that miR-211 could be a good adjunct to the treatment protocol for breast cancer patients if these changes are in fact conserved in vitro.

Our TUNEL assay results demonstrated that miR-211 treatment was associated with a much higher degree of apoptosis in both cell lines. When discussing potential mechanisms by which apoptosis could occur because of miR-211 treatment, several of the factors that miR-211 increased on apoptosis assay have been well established as pro-apoptotic targets in other cancer treatment. TRAIL-R2, also known as death receptor 5, is involved with activating apoptotic pathways, and, when given exogenously, is indicated for increasing immune defense and surveillance against cancer cells. Its monoclonal antibody, tigatuzumab, has been approved for the treatment of ovarian cancer, and has been clinically studied as an adjunct for paclitaxel in triple negative breast cancer [37] as well as in colorectal [38] and non-small cell lung [39] cancer. P21 is a cyclin-dependent kinase inhibitor and is associated with p53 to respond to membrane or DNA damage and promote cell cycle arrest [40]. p27 is another cyclin-dependent kinase inhibitor that acts to decrease proliferation and differentiation of cells by limiting cells' ability to enter S phase. Dephosphorylation of BAD, which is also known as Bcl-2 associated death promoter, is pro-apoptotic. Targeting this protein pharmacologically is currently being studied in pre-clinical trials [41]. Through its induction of apoptosis through these proposed mechanisms, miR-211 treatment created a pro-apoptotic state in both the BT-474 and MDA-MB-468 cell lines. The IPAD assay highlighted how miR-211 affects the phosphorylation status of RB and MAPK proteins, which are known tumor suppressors. These results show miR-211's expression attenuates tumor progression in a multifactorial manner.

In conclusion, we have demonstrated PDK4 is highly expressed in breast cancers irrespective of their molecular or histologic subtype. Furthermore, PDK4's overexpression has a negative impact on prognosis for breast cancer

patients. On the other hand, miR-211 is upregulated in normal breast tissue and downregulated in breast cancer, and that miR-211 is a suppressor of Warburgian aerobic glycolysis. MiR-211 treatment of breast cancer cell lines caused an attenuation in PDK4 expression including in cells treated with ionizing radiation, and was found to increase apoptosis, purportedly by the increased expression of pro-apoptotic factors and alteration of various tumor pathways. These studies elucidate PDK4 as an important target of miR-211 and reveal miR-211 as a robust inhibitor of the Warburg effect and promising therapeutic target for breast cancer treatment.

Acknowledgements

Disclosure of conflict of interest

None.

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Table S1. List of primers with sequences

PDK4-F	GGAAGCATTGATCCTAACTGTGA
PDK4-R	GGTGAGAAGGAACATACACGATG
HIF1-F	GAAAGCGCAAGTCTTCAAAG
HIF1-R	TGGGTAGGAGATGGAGATGC
GAPDH-F	AATCCCATCACCATCTTCCA
GAPDH-R	TGGACTCCACGACGTACTCA
Sox2-F	CAGGAGAACCCCAAGATGCACAA
Sox2-R	AATCCGGGTGCTCCTTCATGTG
TWIST-F	GGACAAGCTGAGCAAGATTCAGA
TWIST-R	TCTGGAGGACCTGGTAGAGGAA
Snail-F	GCTGCAGGACTCTAATCCAGA
Snail-R	ATCTCCGGAGGTGGGATG
hsa-miR-211-F	TTCCCTTTGTCATCCTTCGCCT
Universal Reversal Primer	GTGCAGGGTCCGAGGT
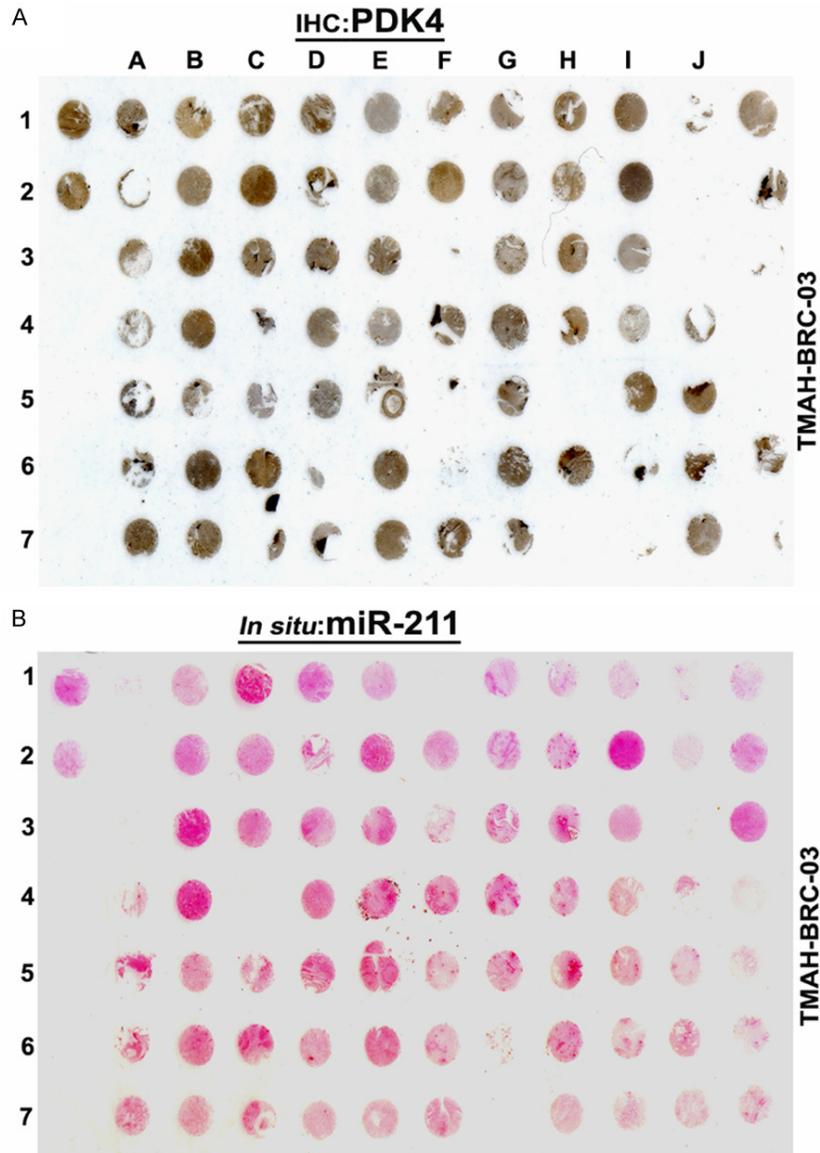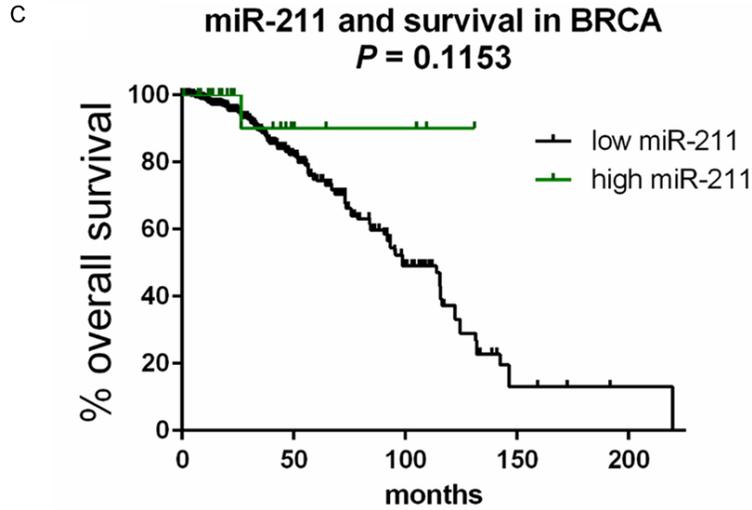
Figure S1. Breast cancer patients show low expression of miR-211. A. Immunohistochemical (IHC) analysis of TMAH-BRC-03 human breast TMA probed for PDK4 expression. B. In situ hybridization assay demonstrating a relative absence of miR-211 RNA in TMAH-BRC-03 TMA. C. Kaplan-Meier survival curve for overall survival of patients with low and high expression of miR-211.
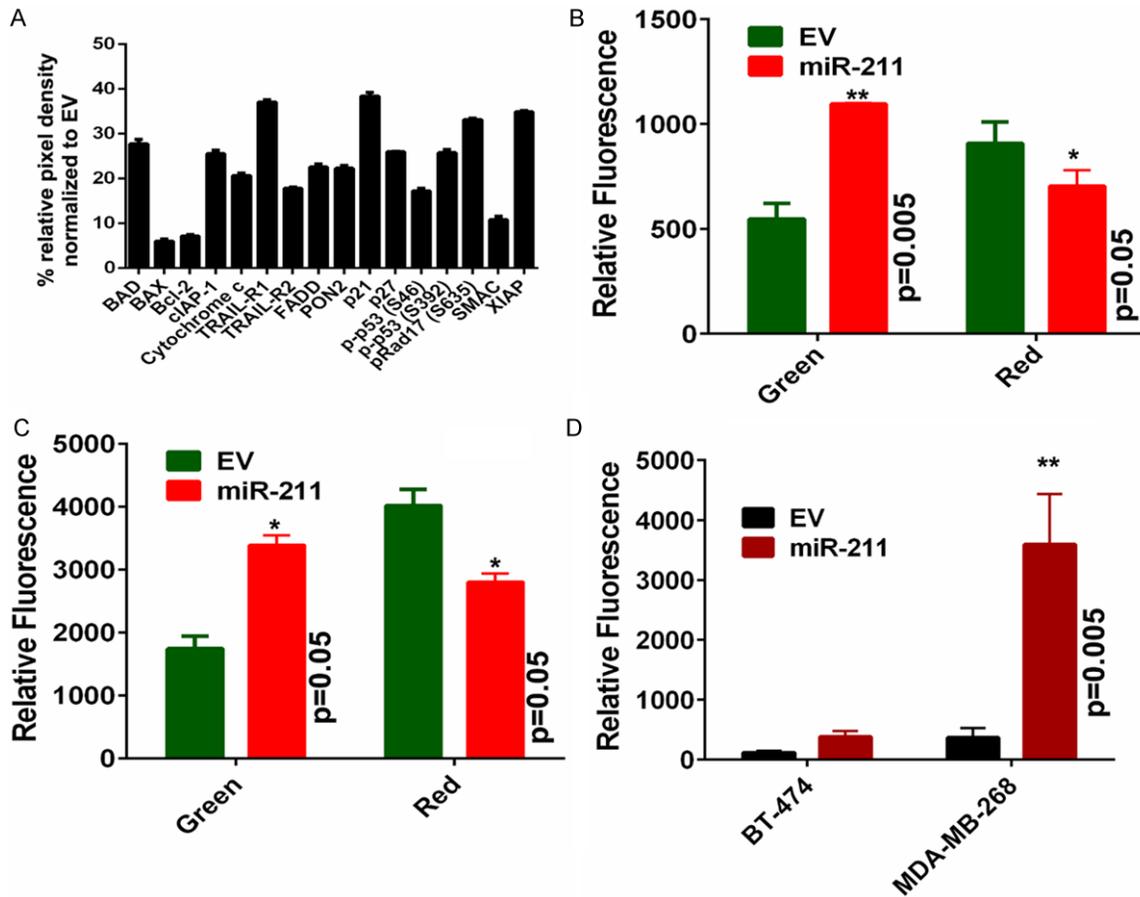
Figure S2. miR-211 overexpression induces apoptosis. A. Quantification of human apoptosis array. The graph is plotted for the apoptotic markers highly expressed in miR-211 treatments compared to the EV. B and C. Quantification of the green fluorescence in the miR-211 treated cells in comparison to red fluorescence of the EV treated cells. Red color suggests the live cells and green color shows the apoptotic cells. D. Quantification of the dead cells using Tunnel assay. The values from control and treated cells were obtained using Image J software.