Cell Cycle 11:23, 4402–4413; December 1, 2012; © 2012 Landes Bioscience

BRCA1 mutations drive oxidative stress and glycolysis in the tumor microenvironment

Implications for breast cancer prevention with antioxidant therapies

Ubaldo E. Martinez-Outschoorn,[1,2,3] Renee Balliet,[1,2] Zhao Lin,[1,2] Diana Whitaker-Menezes,[1,2] Ruth C. Birbe,[1,2] Alessandro Bombonati,[1,2] Stephanos Pavlides,[4] Rebecca Lamb,[4] Sharon Sneddon,[4] Anthony Howell,[4] Federica Sotgia[1,2,4,†,*] and Michael P. Lisanti[1,2,3,4,†,*]

[1]The Jefferson Stem Cell Biology and Regenerative Medicine Center; Kimmel Cancer Center; Thomas Jefferson University; Philadelphia, PA USA; [2]Departments of Stem Cell Biology & Regenerative Medicine, or Pathology, or Cancer Biology; Kimmel Cancer Center; Thomas Jefferson University; Philadelphia, PA USA; [3]Department of Medical Oncology; Kimmel Cancer Center; Thomas Jefferson University; Philadelphia, PA; [4]Manchester Breast Centre & Breakthrough Breast Cancer Research Unit; Paterson Institute for Cancer Research; Institute of Cancer Sciences; Manchester Academic Health Science Centre; University of Manchester; Manchester, UK

[†]Current affiliation: Breakthrough Breast Cancer Research Unit; University of Manchester; Manchester, UK

Keywords: hereditary breast cancer, tumor metabolism, BRCA1 mutations, hydrogen peroxide, oxidative stress, MCT4, caveolin-1 (Cav-1), triple-negative breast cancer, synthetic lethality

Mutations in the BRCA1 tumor suppressor gene are commonly found in hereditary breast cancer. Similarly, downregulation of BRCA1 protein expression is observed in the majority of basal-like breast cancers. Here, we set out to study the effects of BRCA1 mutations on oxidative stress in the tumor microenvironment. To mimic the breast tumor microenvironment, we utilized an in vitro co-culture model of human BRCA1-mutated HCC1937 breast cancer cells and hTERT-immortalized human fibroblasts. Notably, HCC1937 cells induce the generation of hydrogen peroxide in the fibroblast compartment during co-culture, which can be inhibited by genetic complementation with the wild-type BRCA1 gene. Importantly, treatment with powerful antioxidants, such as NAC and Tempol, induces apoptosis in HCC1937 cells, suggesting that microenvironmental oxidative stress supports cancer cell survival. In addition, Tempol treatment increases the apoptotic rates of MDA-MB-231 cells, which have wild-type BRCA1, but share a basal-like breast cancer phenotype with HCC1937 cells. MCT4 is the main exporter of L-lactate out of cells and is a marker for oxidative stress and glycolytic metabolism. Co-culture with HCC1937 cells dramatically induces MCT4 protein expression in fibroblasts, and this can be prevented by either BRCA1 overexpression or by pharmacological treatment with NAC. We next evaluated caveolin-1 (Cav-1) expression in stromal fibroblasts. Loss of Cav-1 is a marker of the cancer-associated fibroblast (CAF) phenotype, which is linked to high stromal glycolysis, and is associated with a poor prognosis in numerous types of human cancers, including breast cancers. Remarkably, HCC1937 cells induce a loss of Cav-1 in adjacent stromal cells during co-culture. Conversely, Cav-1 expression in fibroblasts can be rescued by administration of NAC or by overexpression of BRCA1 in HCC1937 cells. Notably, BRCA1-deficient human breast cancer samples (9 out of 10) also showed a glycolytic stromal phenotype, with intense mitochondrial staining specifically in BRCA1-deficient breast cancer cells. In summary, loss of BRCA1 function leads to hydrogen peroxide generation in both epithelial breast cancer cells and neighboring stromal fibroblasts, and promotes the onset of a reactive glycolytic stroma, with increased MCT4 and decreased Cav-1 expression. Importantly, these metabolic changes can be reversed by antioxidants, which potently induce cancer cell death. Thus, antioxidant therapy appears to be synthetically lethal with a BRCA1-deficiency in breast cancer cells and should be considered for future cancer prevention trials. In this regard, immunostaining with Cav-1 and MCT4 could be used as cost-effective biomarkers to monitor the response to antioxidant therapy.

Introduction

The BRCA1 (breast cancer type 1 susceptibility) gene is a tumor suppressor involved in several important cellular functions, including DNA repair, regulation of transcription, ubiquitination and cell cycle regulation.[1] BRCA1 gene mutations strongly predispose toward the development of breast and ovarian cancers. Female carriers of BRCA1 mutations show a 60–80% lifetime risk of breast cancer and a 40–50% lifetime risk of ovarian cancer.[2,3]

*Correspondence to: Federica Sotgia and Michael P. Lisanti; Email: fsotgia@gmail.com and michaelp.lisanti@gmail.com
Submitted: 10/24/12; Accepted: 11/04/12
http://dx.doi.org/10.4161/cc.22776

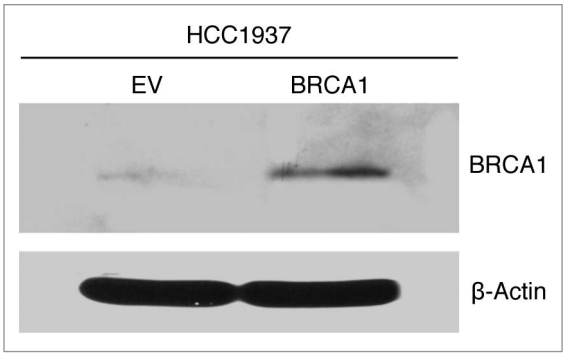

Figure 1. Genetic replacement of wild-type BRCA1 expression in BRCA1-deficient human breast cancer cells. HCC1937 cells with wild-type BRCA1 overexpression were generated using a lentiviral-vector approach. Empty vector (EV) control cells were generated in parallel. Immunoblotting was performed to assess BRCA1 protein expression. HCC1937 cells were cultured for 72 h to achieve confluence. Equal protein loading was assessed by immunoblotting with β-actin. EV, empty vector (Lv105).

Most BRCA1 gene mutations prevent BRCA1 protein production (due to truncating mutations or missense mutations),[4] and most BRCA1-mutated breast cancers show a loss of nuclear BRCA1 expression, which is associated with a worse prognosis.[5,6] BRCA1-mutated breast cancers are most often aggressive, high-grade, aneuploid, triple-negative [ER(-), PR(-) and HER2(-)] and are basal-like, as assessed by gene expression and immunohistochemical analysis.[7-9]

Loss of BRCA1 expression is also very common in sporadic breast cancers, due to epigenetic or post-translational modifications, and is most frequently observed in breast cancers with a basal-like phenotype.[3,10-17] Thus, loss of BRCA1 protein expression is thought to be the driver of the shared phenotype between sporadic basal-like breast cancers and familial breast cancers with BRCA1 mutations. However, the exact mechanism(s) by which loss of BRCA1 function generates a basal-like breast cancer phenotype remains unknown.[18,19]

BRCA1 and oxidative stress. High levels of oxidative stress are associated with aggressiveness in breast cancer[20,21] Several findings indicate that BRCA1 may normally protect against oxidative stress. Overexpression of BRCA1 in breast and prostate cancer cell lines increases the expression of several genes involved in antioxidant responses; for example, glutathione S-transferases, via upregulation of the antioxidant response transcription factor NRF-2,[22] decrease the levels of reactive oxygen species (ROS)[23] and confer resistance to hydrogen peroxide exposure.[22] Conversely, inactivation of BRCA1 induces high levels of oxidative stress, with increased superoxide anion and hydrogen peroxide generation,[24] and sensitizes cells toward oxidative stress, decreasing cell viability.[22] Thus, these results suggest that BRCA1 may function as a natural endogenous antioxidant. However, it remains unknown if drugs with antioxidant properties can efficiently kill BRCA1-mutated cancer cells.

BRCA1 deficiency and the tumor stroma. Metaplastic breast cancers are a subtype of basal-like breast cancers that have a strong fibrotic stromal reaction.[25] Low BRCA1 protein expression due to promoter methylation is found in approximately 70% of metaplastic breast cancers.[10] Conversely, a basal-like cell phenotype is the most common phenotype observed in patients with BRCA1 mutations,[26] and the majority of basal-like breast cancers have a strong stromal reaction, although not sufficient to consider them metaplastic breast cancers.[27] However, little is known about the effects of BRCA1 mutations/loss of function on the tumor stroma.

It is well-described that cancer cells generate high levels of ROS, and that this promotes cancer growth.[28-30] In non-hereditary breast cancer, epithelial breast cancer cells compel surrounding stromal cells to generate more ROS, leading to an oxidative stress cascade.[31] Elevated ROS levels induce glycolytic metabolism in the tumor stroma, with increased expression of the L-lactate exporter MCT4, which further promotes tumor growth.[31-34] We have previously shown that high expression of MCT4 in the tumor stroma predicts poor clinical outcome in patients with triple-negative breast cancers.[35] We have also demonstrated that the antioxidant N-acetyl cysteine (NAC) can downregulate the expression of MCT4 in cancer-associated fibroblasts.[32] Here, we set out to investigate if BRCA mutations in epithelial breast cancer cells can modulate the generation of ROS and glycolytic metabolism in adjacent stromal cells.

Results

BRCA1 rescue decreases hydrogen peroxide generation in BRCA1-null epithelial breast cancer cells (HCC1937) co-cultured with fibroblasts. To investigate the relationship between BRCA1 mutations, oxidative stress and epithelial-stromal interactions, we conducted in vitro co-culture experiments with HCC1937 human breast cancer cells (carrying BRCA1 mutations) and hTERT-immortalized BJ1 fibroblasts. First, as a critical experimental control, we rescued BRCA1 expression in HCC1937 cells, using a lentiviral vector approach (HCC1937 + BRCA1 cells), and demonstrated BRCA1 re-expression by immunoblotting (**Fig. 1**). Then, empty vector control (EV) or BRCA1-overexpressing HCC1937 cells were co-cultured with fibroblasts to measure hydrogen peroxide production. Under these co-culture conditions, BRCA1 overexpression decreases hydrogen peroxide generation in epithelial HCC1937 cells by 1.7-fold (**Fig. 2**). However, no significant differences were noted during homotypic culture (data not shown).

BRCA1-null breast cancer cells also promote hydrogen peroxide generation in adjacent stromal fibroblasts during co-culture, which is blunted by BRCA1 re-expression in the epithelial cell compartment. We next evaluated if BRCA1-null breast cancer cells induce hydrogen peroxide generation in adjacent stromal fibroblasts during co-culture. **Figure 3** shows that HCC1937 cells lead to a 1.8-fold increase in hydrogen peroxide generation in co-cultured fibroblasts, as compared with homotypic fibroblast cultures. Importantly, this stromal fibroblast phenotype is rescued by the overexpression of wild-type BRCA1 in HCC1937 cells (**Fig. 3**).

Treatment with the antioxidants NAC and Tempol induces the apoptotic death of BRCA1-null breast cancer cells. Having

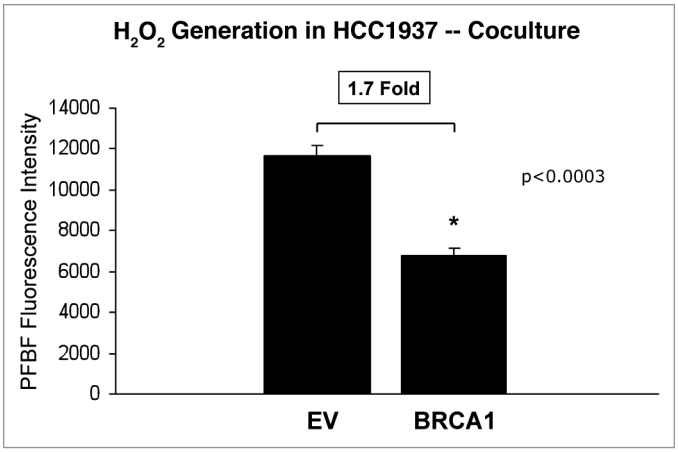

Figure 2. BRCA1 rescue decreases hydrogen peroxide generation in HCC1937 cells during co-culture with fibroblasts. Empty vector (EV) control HCC1937 cells and BRCA1-overexpressing HCC1937 cells were cultured with BJ1-RFP fibroblasts for 7 d, and H_2O_2 levels were measured using PFBS-F. Note that H_2O_2 generation decreases by 1.7-fold in BRCA1-overexpressing cells, as compared with control HCC1937 cells ($p < 0.0003$). EV, empty vector (Lv105).

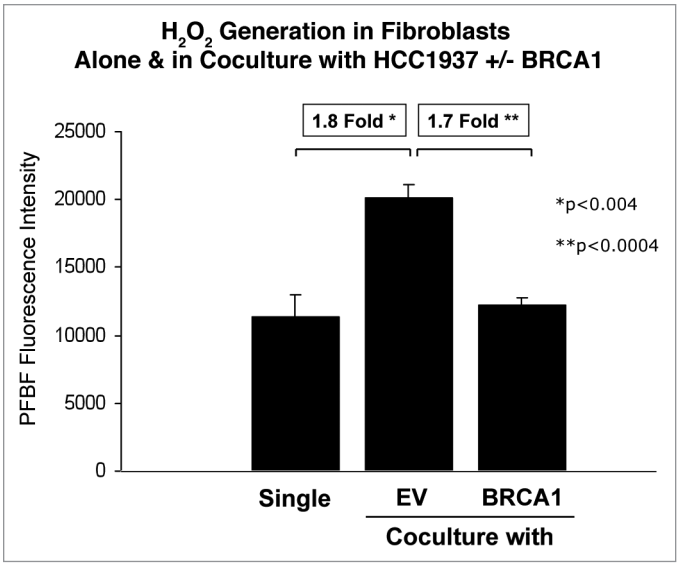

Figure 3. BRCA1-null breast cancer cells (HCC1937) induce hydrogen peroxide generation in adjacent fibroblasts. BJ1-RFP fibroblasts were cultured alone or co-cultured with control HCC1937 cells or with HCC1937 + BRCA1 cells for 7 d, and H_2O_2 levels were measured using PFBS-F. Note that H_2O_2 generation increases by 1.8-fold in BJ1 fibroblasts during co-culture with HCC1937 cells ($p < 0.004$), relative to fibroblasts alone. However, co-culture with HCC1937 + BRCA1 cells decreases H_2O_2 generation in fibroblasts by 1.7-fold, as compared with co-culture with control HCC1937 cells ($p < 0.0004$), and normalizes H_2O_2 generation to similar levels as seen in fibroblasts alone. EV, empty vector (Lv105).

found a link between BRCA1 and ROS generation, we set out to determine the effects of the antioxidant NAC on the apoptotic rates of HCC1937 cells in homotypic culture and during co-culture with fibroblasts. Interestingly, NAC increases the apoptosis of HCC1937 cells by 3.6-fold in homotypic culture (**Fig. 4A**) and by 1.4-fold during co-culture with fibroblasts (**Fig. 4B**). We next studied the effect of the nitroxide antioxidant Tempol on apoptosis in HCC1937 cells. Tempol demonstrates a dose-response effect on the apoptosis rates of HCC1937 cells under homotypic and co-culture conditions (**Fig. 5**). Remarkably, treatment with 7.5 mM Tempol potently induces the apoptosis of > 97% HCC1937 cells during both homotypic cultures (**Fig. 5A**) and co-culture (**Fig. 5B**). Thus, a BRCA1 deficiency appears to be synthetically lethal with antioxidant therapy.

Tempol treatment induces apoptotic cell death in a triple-negative breast cancer cell line (MDA-MB-231 cells), but only during co-culture with fibroblasts. Due to the many similarities between BRCA1-mutated breast cancer and basal-like breast cancer, and having found that the antioxidant Tempol potently induces apoptosis of BRCA1-mutated cancer cells, we set out to determine the effects of Tempol on apoptosis of the basal-like breast cancer cell line MDA-MB-231. Similarly to HCC19137 cells, Tempol has a dose-response effect on apoptosis rates in MDA-MB-231 cells under co-culture conditions. Apoptosis rates of MDA-MB-231 cells increase by 2.3-fold, from 30.9% for untreated cells to 70.7% for 7.5mM Tempol ($p < 9.7E-6$) (**Fig. 6A**). Surprisingly, addition of Tempol did not significantly increase apoptosis in homotypic cultures of MDA-MB-231 cells (**Fig. 6B**). Thus, Tempol only kills MDA-MB-231 breast cancer cells during their co-culture with fibroblasts.

BRCA1-null breast cancer cells induce MCT4 expression in adjacent stromal fibroblasts: Rescue with wild-type BRCA1 or antioxidant treatment. MCT4 is the main transporter of L-lactate out of cells and is a marker of oxidative stress and glycolytic metabolism. Thus, we next assessed MCT4 expression under both homotypic and co-culture conditions. As predicted, co-culture with HCC1937 cells leads to the upregulation of MCT4 expression in stromal fibroblasts (**Fig. 7A**), indicative of the onset of oxidative stress and a glycolytic phenotype. Conversely, treatment with the antioxidant NAC (10 mM) prevents the induction of MCT4 expression in stromal fibroblasts during co-culture (**Fig. 7A**).

We next asked if epithelial rescue of BRCA1 expression can also block the stromal induction of MCT4. Thus, fibroblasts were co-cultured with HCC19137 cells overexpressing BRCA1. For comparison, fibroblast-HCC19137 cell co-cultures were also treated with NAC. Note that MCT4 expression is highest in fibroblasts co-cultured with BRCA1-null HCC1937 cells, as compared with all the other conditions tested (**Fig. 7B**). Most importantly, co-culture with HCC1937 + BRCA1 cells or addition of NAC prevents the induction of MCT4 expression in stromal fibroblasts (**Fig. 7B**).

Co-culture with BRCA1-null breast cancer cells drives a loss of Cav-1 expression in stromal fibroblasts: Rescue by antioxidant treatment or via epithelial re-expression of wild-type BRCA1. We next focused our attention on caveolin-1 (Cav-1) expression in fibroblasts, since a loss of stromal Cav-1 expression is associated with aggressive triple-negative breast cancer.[35-37] Mechanistically, stromal Cav-1 is downregulated in fibroblasts during oxidative stress, as it is targeted to the lysosome for its autophagic degradation; thus, a loss of stromal Cav-1

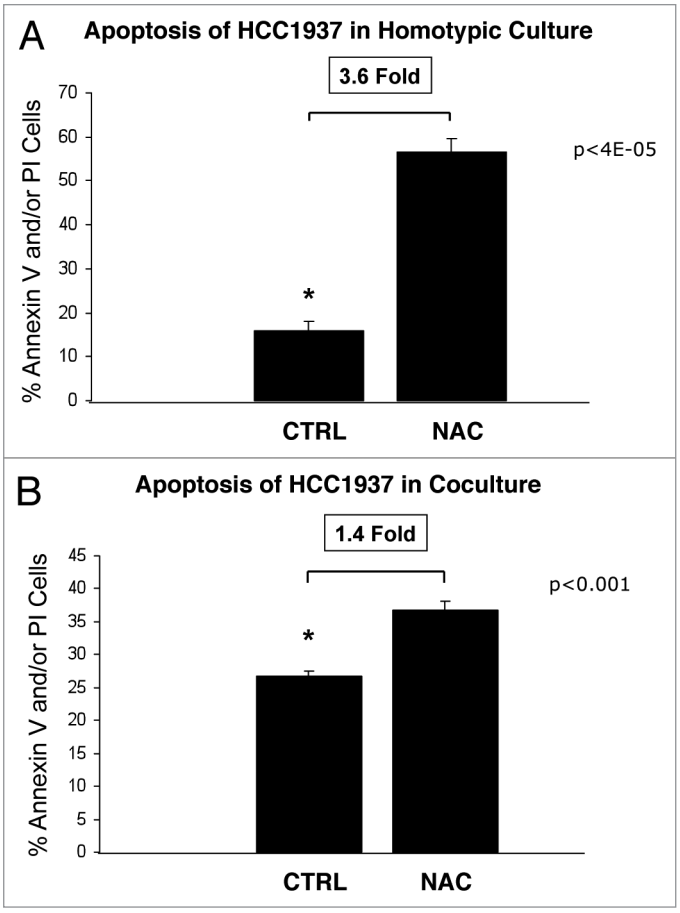

Figure 4. The antioxidant NAC induces apoptotic cell death in BRCA-null human breast cancer cells. Homotypic HCC1937 cell culture and co-culture with GFP fibroblasts was performed for 48 h. Then, vehicle or 10 mM NAC was added to the cell culture media 48 h prior to measurement of apoptosis with annexin V-APC and PI. Six hours prior to measuring apoptosis, the media was changed to HBSS with and without 10 mM NAC. Note that NAC increases the apoptotic rates of HCC1937 cells by 3.6-fold ($p < 4E-5$) in homotypic culture (**A**), and by 1.4-fold in co-culture (**B**) ($p < 0.001$).

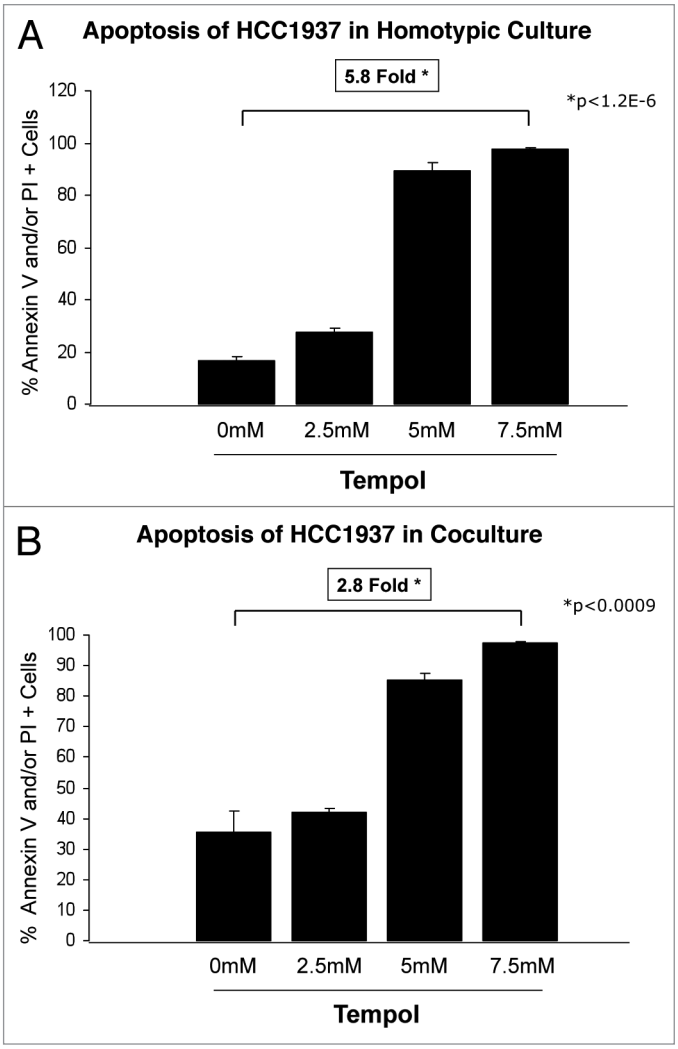

Figure 5. Treatment with the antioxidant Tempol is lethal in BRCA1-null human breast cancer cells. Homotypic HCC1937 cell culture and co-culture with GFP fibroblasts was performed for 24 h. Vehicle or 2.5 mM, 5 mM, 7.5 mM Tempol was added to cell culture media 24 h prior to measurement of apoptosis with annexin V-APC and PI. Note that Tempol induces apoptosis in HCC1937 in a dose-dependent fashion in homotypic culture (**A**), with 97.4% of cells undergoing apoptosis at 7.5 mM Tempol ($p < 1.2E-6$). Similarly, Tempol induces apoptosis in HCC1937 during co-culture (**B**), with > 97.5% apoptosis after treatment with 7.5 mM Tempol ($p < 0.009$).

is a functional marker for oxidative stress and autophagy in the tumor microenvironment.[33,38,39,40]

First, we evaluated Cav-1 expression in fibroblasts during co-culture with HCC1937 cells. Note that co-culture with HCC1937 cells leads to a loss of Cav-1 in stromal fibroblasts, as compared with fibroblasts cultured alone, and this can be rescued by antioxidant treatment with NAC (**Fig. 8A**). However, NAC treatment has no effect on Cav-1 expression in fibroblasts cultured homotypically. Finally, co-culture with HCC1937 + BRCA1 cells also rescues Cav-1 expression in stromal fibroblasts in a similar fashion to NAC (**Fig. 8B**). Thus, antioxidant therapy, or BRCA1 rescue in the epithelial cancer cell compartment, prevents the loss of Cav-1 in the stromal fibroblast compartment.

Primary tumor samples from BRCA1-mutated breast cancer patients show a loss of Cav-1 and high MCT4 in the tumor stroma, with increased mitochondrial mass in BRCA1-deficient epithelial cancer cells. We next assessed the expression of Cav-1 and MCT4 in human BRCA1-mutated breast cancer samples. Remarkably, in nine out of 10 BRCA1-mutated patients, tumor-associated stromal fibroblasts show low or absent expression of Cav-1 and high expression of MCT4 (**Fig. 9,** upper and middle panels). Conversely, BRCA1-mutated breast cancer epithelial cells have low MCT4 expression, as compared with that observed in fibroblasts (**Fig. 9,** middle panels).

We also assessed mitochondrial mass in BRCA1-mutated breast cancer samples. TOMM20 is a mitochondrial membrane protein involved in the import of proteins into the mitochondria. Studies have shown that expression of TOMM20 correlates with oxidative mitochondrial metabolism (OXPHOS). Note that tumor-associated fibroblasts have low TOMM20

expression, while BRCA1-mutated epithelial cancer cells have high TOMM20 expression (**Fig. 9,** lower panels).

In summary, BRCA1-mutated epithelial cancer cells show high expression of a marker of mitochondrial mass, while fibroblasts show expression of markers of glycolysis and oxidative stress. These results indicate that our co-culture model of HCC1937 cells and stromal fibroblasts directly recapitulates the microenvironmental features of BRCA1-mutated breast cancer patients.

Discussion

The aim of the current study was to evaluate the effects of BRCA1 mutations on oxidative stress and glycolysis in the tumor microenvironment. To this end, we utilized the human BRCA1-mutated breast cancer cell line HCC1937- and hTERT-immortalized fibroblasts in an in vitro co-culture model of breast cancer. Notably, BRCA1-mutated HCC1937 cells induce the generation of hydrogen peroxide in the fibroblast compartment during co-culture, and BRCA1 re-expression in HCC1937 epithelial cancer cells is sufficient to blunt the oxidative stress reaction in adjacent stromal cells. Importantly, treatment with antioxidants potently induces apoptotic cell death in BRCA1-mutated and basal-like cancer cells. In addition, we show that BRCA1-mutated breast cancer cells drive the onset of a glycolytic tumor microenvironment, as assessed by elevated expression of MCT4 and a loss of Cav-1. Analysis of primary breast cancer samples from patients with BRCA1-mutations clearly validates our in vitro findings, and indicates that the onset of stromal-epithelial metabolic coupling supports aggressive cancer behavior (**Fig. 10**).

Antioxidants phenocopy the effects of BRCA1 rescue in BRCA1-null mammary epithelial cancer cells. To our knowledge, this is the first study to evaluate the effects of the antioxidants NAC and Tempol on the human BRCA1-mutated HCC1937 cell line. We also evaluated the effects of antioxidants on the human basal-like breast cancer cell line MDA-MB-231 with wild-type BRCA1, but carrying post-translational modifications that alter its promoter binding.[11,41] We show that both antioxidants induce the apoptosis of BRCA1-mutated HCC1937 cells, and that Tempol also induces apoptosis in the basal-like MDA-MB-231 cells.

Mechanistically, we show that NAC suppresses the induction of MCT4 expression in the stromal fibroblast compartment during co-culture with BRCA1-mutated HCC1937 cells, indicating that high hydrogen peroxide generation in BRCA1-null breast cancer cells drives MCT4 expression in fibroblasts. MCT4 is the main transporter of L-lactate out of cells and is a marker of oxidative stress and glycolytic metabolism.[42] Importantly, high expression of MCT4 in the tumor stroma predicts poor clinical outcome in triple-negative breast cancer.[35] We have previously shown that glycolysis in the tumor stroma supports and promotes breast cancer tumor growth and progression.[33,34,43,44]

We show also that BRCA1-mutated cancer cells induce a loss of Cav-1 in fibroblasts, and this is can also be reverted by treatment with antioxidants. A loss of Cav-1 in cancer-associated fibroblasts is associated with poor prognosis in many tumor types, including breast cancer.[45-48] Studies have shown that fibroblasts with a loss of Cav-1 generate high levels of ROS, have high levels of autophagy and glycolytic metabolism, and these appear to be the mechanism(s) that drive tumor progression.[31,33,38,45] Importantly, genetic complementation of BRCA1 exerts similar effects as treatment with antioxidants, by restoring Cav-1 stromal expression and inhibiting MCT4 stromal upregulation. These results indicate that BRCA1 normally functions as an endogenous antioxidant and, most importantly, suggests that antioxidant treatment may be a sensible alternative to BRCA1 gene therapy.

Antioxidants as an option for the treatment and/or prevention of BRCA1-null and basal-like breast cancers. We show here that the use of the antioxidant NAC can reverse glycolytic

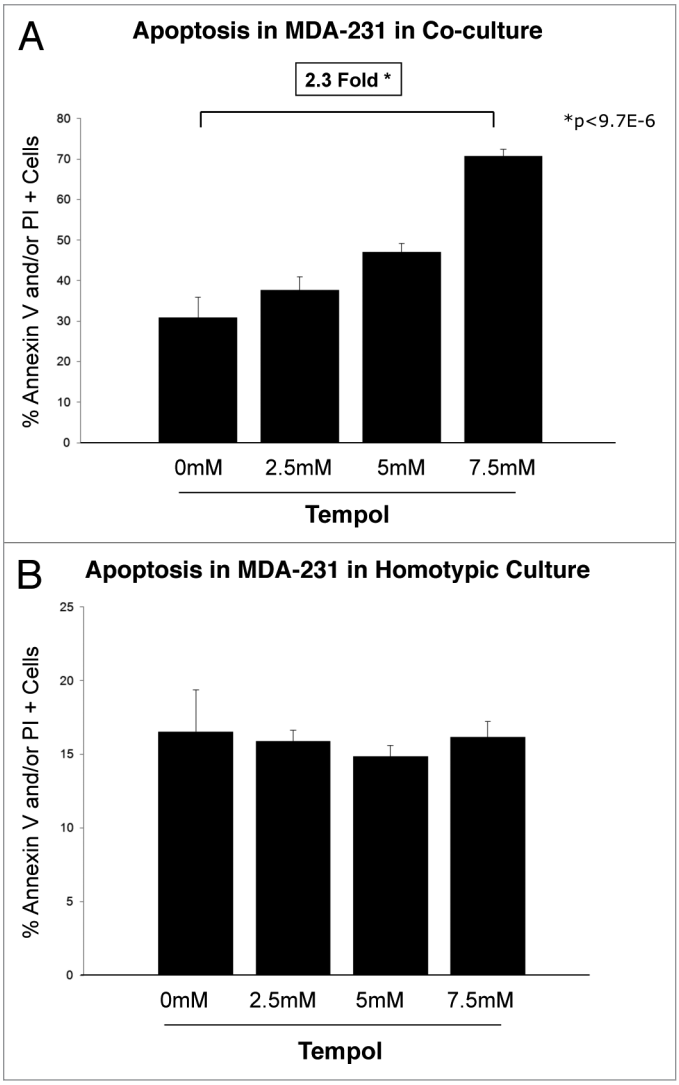

Figure 6. The antioxidant Tempol induces apoptosis in triple-negative breast cancer cells (MDA-MB-231), but only during co-culture with fibroblasts. Homotypic MDA-MB-231 cell culture and co-culture with GFP fibroblasts was performed for 24 h. Vehicle or 2.5 mM, 5 mM, 7.5 mM Tempol was added to cell culture media 24 h prior to the measurement of apoptosis with annexin V-APC and PI. Note that 7.5 mM Tempol induces a 2.3-fold increase in the apoptosis rates of co-cultured MDA-MB-231 cells, as compared with untreated cells (**A**). Remarkably, Tempol exerts no effects on the apoptosis rates of MDA-MB-231 cells in homotypic culture (**B**).

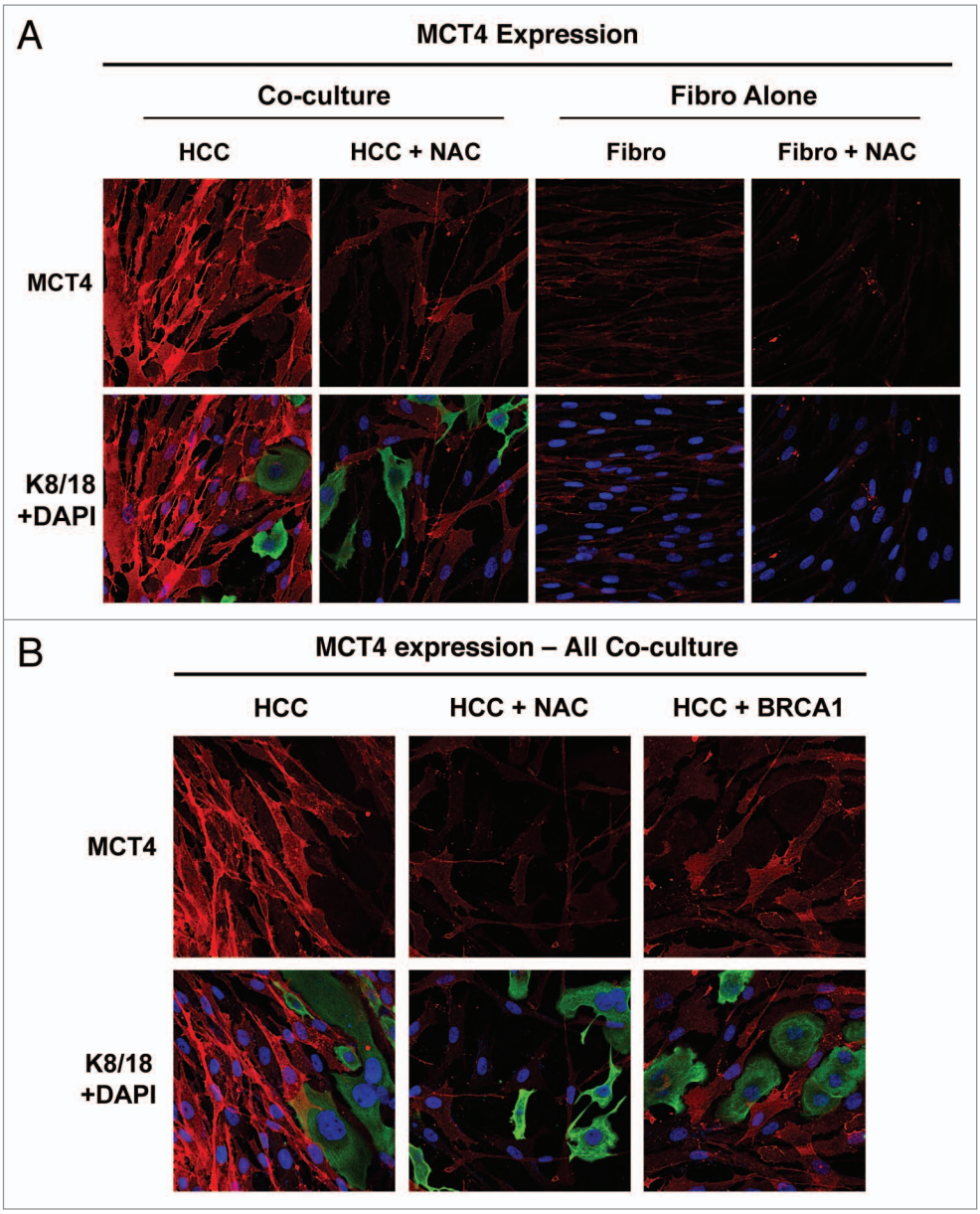

Figure 7. MCT4 expression is induced in stromal fibroblasts during co-culture with BRCA1-null breast cancer cells: Rescue with antioxidant treatment or via BRCA1 overexpression in epithelial cancer cells. (**A**) BJ1 fibroblasts were cultured alone or with HCC1937 cells for 5 d. Then, 10 mM NAC or vehicle was added every 24 h for 48 h. (**B**) HCC1937 cells or HCC1937 + BRCA1 cells were cultured with BJ1 fibroblasts for 5 d. Then, 10 mM NAC or vehicle was added every 24 h for 48 h. For both panels (**A**) and (**B**), the cells were fixed and immunostained with anti-MCT4 and anti-K8/18 antibodies. MCT4 staining (red) is shown in the top panels, and keratin (green) and DAPI (blue) are shown in the bottom panels. (**A**) MCT4 expression is highly induced in fibroblasts during co-culture with HCC1937 cells, and this is abolished by NAC treatment. (**B**) Co-culture with HCC1937 + BRCA1 cells prevents the up-regulation of MCT4 in fibroblasts, as compared with co-culture with HCC1937 cells, in a similar fashion to treatment with the antioxidant NAC. Original magnification, 40x.

metabolism in stromal cells by downregulating MCT4. We also demonstrate that antioxidants, such as NAC and Tempol, induce apoptosis in BRCA1 null and basal-like breast cancer cells in an in vitro model that includes the tumor microenvironment. Thus, antioxidant-induced apoptosis of epithelial cancer cells is linked to reversal of glycolysis in stromal cells, with downregulation of MCT4. Having shown an association between high MCT4 expression in the stroma and induction of epithelial cancer cell apoptosis with antioxidants, it will be important to determine if MCT4 expression in the tumor stroma can be used as a new biomarker to assess breast cancer responsiveness to antioxidant therapy.

The use of antioxidants in the treatment of BRCA1-mutated and basal-like breast cancers may be important, since morbidity and mortality is extremely high in this setting.[19] Basal-like and BRCA1-mutated breast cancer are very aggressive, frequently fatal, commonly associated with new contralateral breast cancers and are difficult to detect by conventional screening

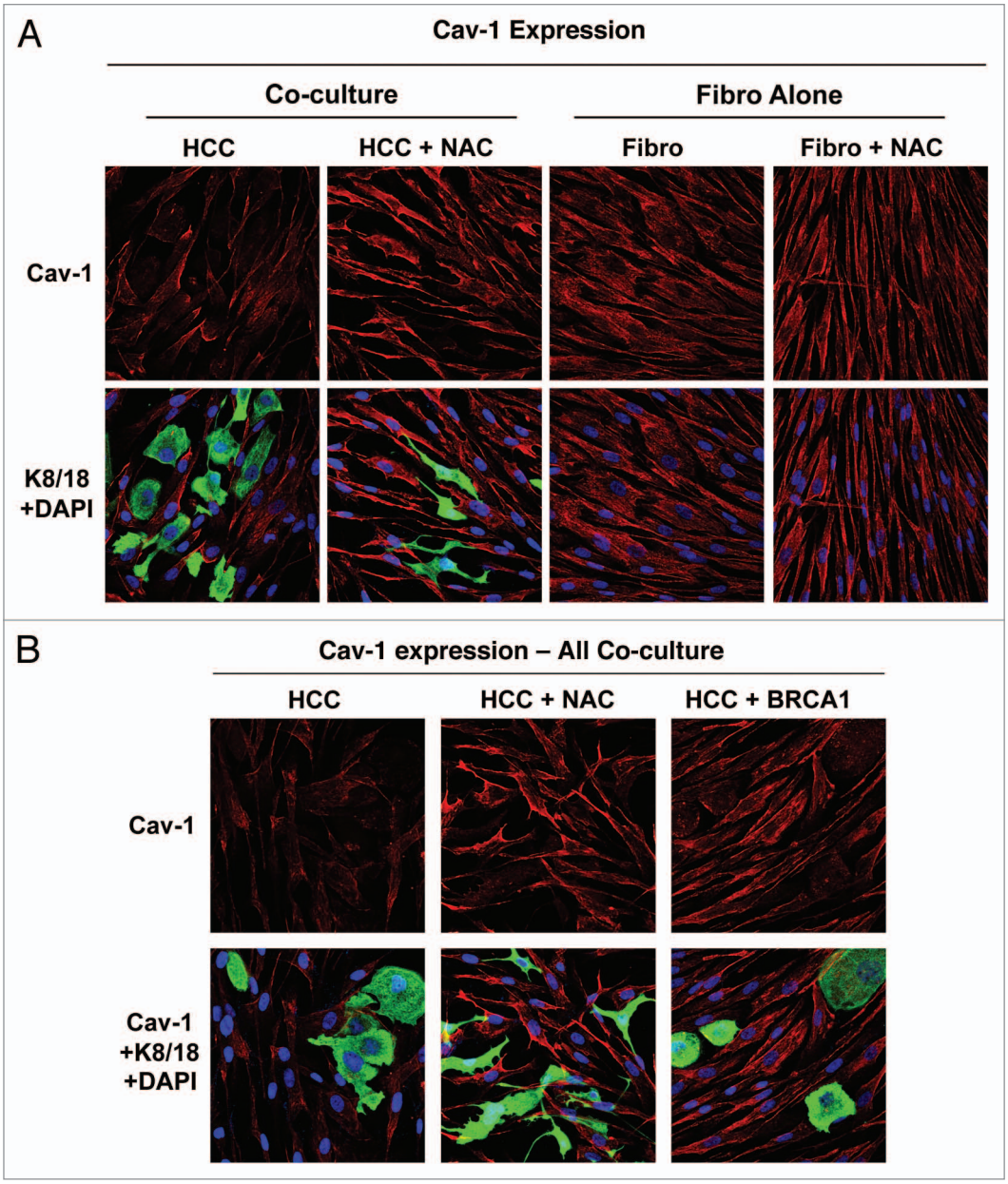

Figure 8. Cav-1 expression is downregulated in stromal fibroblasts co-cultured with BRCA1-null breast cancer cells: Rescue with antioxidant treatment or via BRCA1 overexpression in epithelial cancer cells. (**A**) BJ1 fibroblasts were cultured alone or with HCC1937 cells. Then, 10 mM NAC or vehicle was added every 24 h for 48 h. (**B**) BJ1 fibroblasts were co-cultured with HCC1937 cells or HCC1937 + BRCA1 cells for 5 d. Then, 10 mM NAC or vehicle alone was added every 24 h for 48 h. For both panels (**A**) and (**B**), the cells were fixed and immunostained with anti-Cav-1 and anti-K8–18 antibodies. Cav-1 staining (red) is shown in the top panels and keratin (green) and DAPI (blue) are shown in the bottom panels. Note the loss of Cav-1 expression in stromal fibroblasts co-cultured with HCC1937 cells (**A**). Cav-1 expression can be rescued by NAC treatment (**A**) or by BRCA1 re-expression, using HCC1937 + BRCA1 cells (**B**). The addition of NAC has no effect on fibroblasts during homotypic culture (**A**) or in co-culture with HCC1937 + BRCA1 cells (not shown). Original magnification, 40x.

methods.[19,49,50] Basal-like sporadic and BRCA1-mutated breast cancers have increased sensitivity to oxidative stress via radiation but frequently relapse shortly after treatment.[51-53] Instead of treating basal-like breast cancers by inducing oxidative stress via radiation therapy, an opposite treatment strategy with antioxidants may decrease relapses.

No treatments are currently available to restore BRCA1 function in subjects with BRCA1 mutations. The only preventive treatments in this setting are prophylactic bilateral mastectomies and oophorectomies. These surgeries improve outcomes for BRCA1 mutation carriers, although the morbidity and the effects on quality of life are significant.[54-56] Treatment with antioxidants may be an alternative cancer prevention strategy in subjects with BRCA1 mutations. NAC, resveratrol and selenium show promise as prophylaxis for subjects with BRCA1 mutations, since they reduce ROS levels in BRCA1-mutated cells.[57-59]

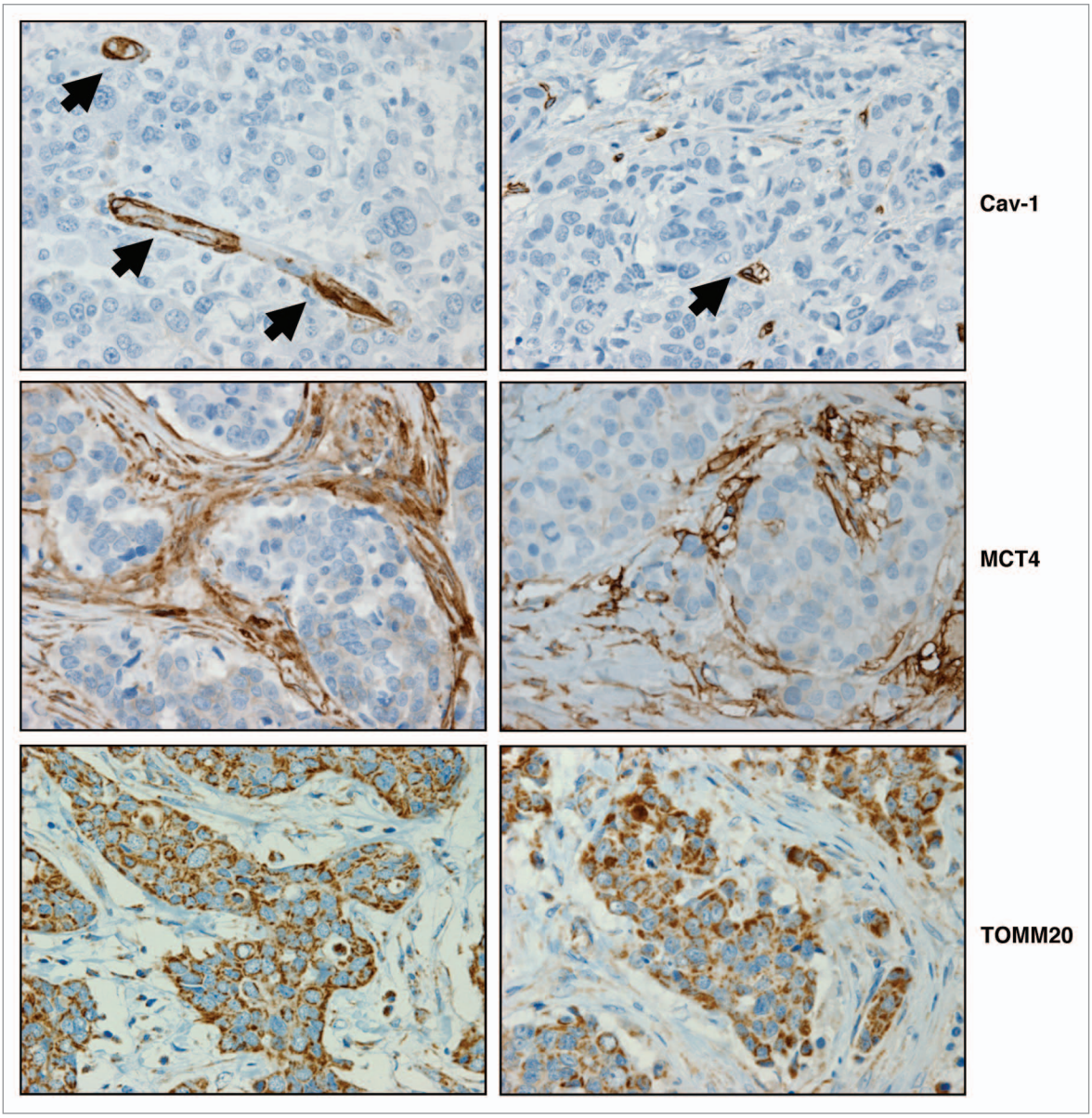

Figure 9. BRCA1-mutated breast cancer samples (from 9 out of 10 patients examined) also show a loss of Cav-1 and high MCT4 expression in cancer-associated fibroblasts, with elevated mitochondrial mass in the epithelial cancer cell compartment. Human breast cancer samples from BRCA1-mutated patients were immunostained with anti-Cav-1, MCT4 or TOMM20 antibodies. Note that stromal cells display the loss of Cav-1 expression, with high MCT4 expression. Cav-1 expression in endothelial cells serves as an internal positive control (arrows), as it is not normally downregulated. Conversely, epithelial cancer cells show very high TOMM20 expression relative to adjacent stromal tissue. TOMM20 is a well-established marker of mitochondrial mass. These observations are indicative of the onset of oxidative stress, autophagy and glycolysis in the tumor microenvironment, with enhanced mitochondrial biogenesis in BRCA1-mutated breast cancer cells. Consistent with the idea that the tumor stroma is glycolytic, the cancer-associated fibroblasts are MCT4(+) and TOMM20(-). Conversely, the BRCA1-mutated epithelial cancer cells are MCT4(-) and TOMM20(+), reflective of oxidative mitochondrial metabolism. Finally, loss of stromal Cav-1 staining is an established marker of autophagy in the tumor microenvironment. Original magnification, 40x.

Also, our results indicate that oxidative stress may be the driver of the characteristic breast cancer phenotype in BRCA1 mutation carriers. BRCA1 mutation carriers develop predominantly triple-negative breast cancers, with a basal-like or myoepithelial cell phenotype, with high proliferation rates and heavy inflammatory cell infiltration.[8,60] It will be important to determine if the aggressive phenotype can be ameliorated by suppressing oxidative stress using antioxidants.

Limitation of using breast cancer cells with multiple mutations other than BRCA1. HCC1937 cells are homozygous for the 5382C insertion mutation in the BRCA1 gene but carry additional mutations in the TP53 and PTEN genes and are polyploid with multiple cytogenetic abnormalities.[41,61] One could argue that it is difficult to determine the effect of BRCA1 mutations on oxidative stress and stromal metabolism when multiple genetic abnormalities are present in the epithelial cancer cells. However, we believe

that in our experimental approach, this limitation is overcome by the overexpression of wild-type BRCA1 in HCC1937 cells, which effectively functions as an antioxidant. Because of their highly mutated status, HCC1937 cells recapitulate the salient features of human BRCA1-mutated breast cancers, which are high aggressiveness, with aneuploidy and the presence of numerous mutations. The use of BRCA1 recombinant overexpression and co-culture with fibroblasts is a significant strength of our study, since this experimental approach has allowed us to directly show that wild-type BRCA1 normally maintains oxidative stress at low levels by functioning as a natural endogenous antioxidant.

Experimental Procedures

Materials. Antibodies were as follows: BRCA1 (CS9010, Cell Signaling), MCT4 (gift of Dr. Nancy Philp, which are isoform-specific rabbit polyclonal antibodies against the 18-mer synthetic oligopeptide corresponding to the C-terminal amino acids of MCT4[62]), Keratin 8/18 (20R-CP004, Fitzgerald Industries International), caveolin-1 (sc-894, Santa Cruz Biotech), TOMM20 (sc-17764, Santa Cruz Biotech) and β-actin (A5441, Sigma). Secondary antibodies for immunofluorescence and flow cytometry were Alexa Green 488nm and Alexa Orange-Red 546nm (Invitrogen). Other reagents were as follows: N-acetyl cysteine (NAC) from Sigma, Tempol from EMD Millipore, 4,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) from Invitrogen, Annexin V-APC conjugate from BD Biosciences, and PFBS-F (Pentafluorobenzenesulfonyl fluorescein) from Cayman Chemicals.

Cell cultures. The breast cancer cell lines HCC1937 and MDA-MB-231 were purchased from ATCC, and HCC1937 + BRCA1 cells were generated by stable overexpression of wild-type BRCA1. HCC1937 cells carry a frameshift mutation in BRCA1 that leads to a BRCA1-null genotype, which has been extensively characterized.[41] Human skin fibroblasts immortalized with telomerase reverse transcriptase protein (hTERT-BJ1) were originally purchased from Clontech, Inc., and stable transfectants that were "color-coded" were generated via either GFP or RFP overexpression. Cells were cultured in DMEM, 10% FBS with Penicillin 100 units/mL-Streptomycin 100 μg/mL.

Stable overexpression of wild-type BRCA1 in HCC1937 cells. The BRCA1 (EX-H0047-Lv105) and control (EX-NEG-Lv105) vectors were purchased from GeneCopoeia, and lentiviruses were prepared according to the manufacturer's protocol. Virus-containing media was centrifuged, filtered (0.45 μM PES low protein filter) and stored in 1-ml aliquots at -80°C. HCC1937 cells (120,000 cells/well) were plated in 12-well dishes in growth media. After 24 h, media was removed and replaced with 250 μl DMEM + 5% FBS, 150 μl virus-containing media and 5 μg/ml polybrene. Twenty-four hours post transfection, media containing virus was removed and replaced with DMEM with 10% FBS. Then, cells were selected with 1 μM puromycin for 5 d to generate HCC1937 cells with stable overexpression of wild-type BRCA1.

Co-cultures of breast cancer cells and fibroblasts. BJ1 fibroblasts (overexpressing GFP or RFP) and breast cancer cell lines

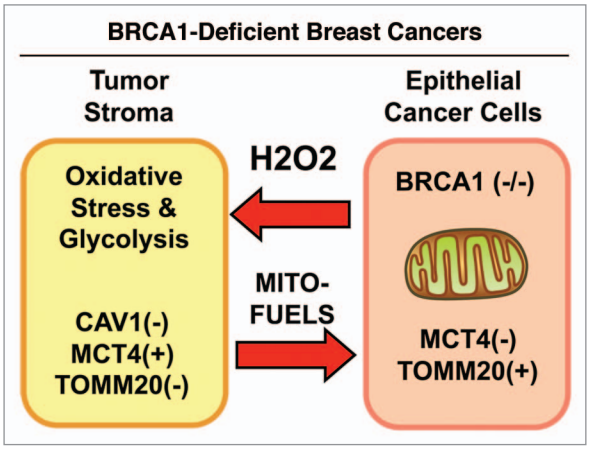

Figure 10. BRCA1-null human breast cancer cells establish two-compartment tumor metabolism. Here, we show that BRCA1 normally functions as an endogenous antioxidant, which helps prevents the onset of hereditary breast cancers. More specifically, BRCA1-deficient epithelial cancer cells produce large amounts of hydrogen peroxide, which functionally induces oxidative stress and glycolysis in the tumor microenvironment. In turn, these glycolytic cancer-associated fibroblasts then provide mitochondrial fuels (such as L-lactate) to epithelial cancer cells, to burn via oxidative mitochondrial metabolism (OXPHOS). Importantly, this "metabolic symbiosis" phenotype is reversed by genetic replacement of the wild-type BRCA1 gene in epithelial cancer cells, or by the administration of antioxidants (NAC, Tempol), providing a new treatment strategy for cancer prevention. In accordance with our mechanistic findings, human breast primary tumor samples from patients with BRCA1 mutations also show a glycolytic/autophagic tumor stroma, as evidenced by their marker status: Cav-1 (-), MCT4(+), and TOMM20 (-). Conversely, BRCA1-mutated epithelial cells are MCT4(-), and TOMM20(+), consistent with mitochondrial biogenesis and oxidative metabolism. Thus, a glycolytic microenvironment surrounds mitochondrial-rich epithelial cancer cells, establishing two metabolic compartments that co-exist, side-by-side. Loss of Cav-1 is a marker of stromal autophagy. Increased MCT4 is an indicator of increased oxidative stress, glycolysis and mitochondrial dysfunction, and increased TOMM20 is associated with mitochondrial biogenesis as well as oxidative mitochondrial metabolism.

(HCC1937 or HCC1937 + BRCA1 or MDA-MB-231) were plated onto glass coverslips in 12-well plates in 1 ml of complete media. Epithelial cells were plated within 2 h of fibroblast plating. The total number of cells per well was 1×10^5. Experiments were performed at a 5:1 fibroblast-to-epithelial cell ratio. As controls, monocultures of fibroblasts and epithelial cells were seeded using the same number of cells as the corresponding co-cultures. The day after plating, media was changed to DMEM with 10% NuSerum (a low serum alternative to FBS, BD Biosciences), and Pen-Strep. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Immunoblotting. This was performed as previously described.[39] Briefly, cells were scraped in urea lysis buffer (6.7 M urea, 10% glycerol, 1% SDS, 10 mM TRIS-HCl pH 6.6, 1% Triton X-100, protease inhibitors), homogenized for 5 sec and incubated on ice for 10 min. After centrifugation, protein concentration was determined using the Bradford assay (BioRad). Thirty μg of proteins were loaded and separated by SDS-PAGE and transferred to a 0.2 μm nitrocellulose membrane (Fisher

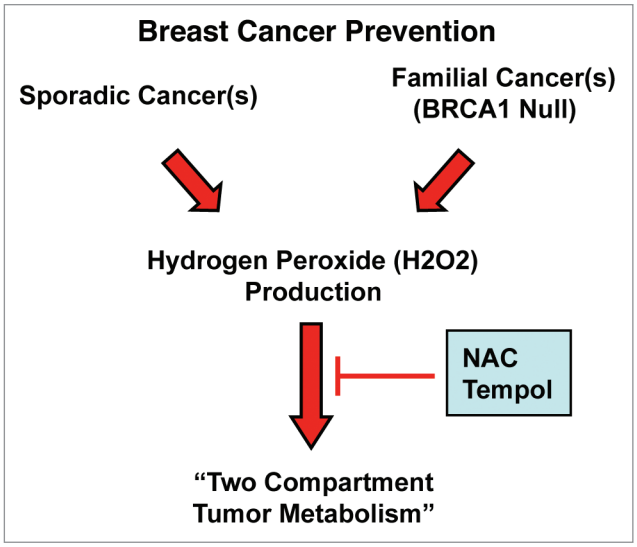

Figure 11. Sporadic and hereditary breast cancers use the same convergent mechanism(s): Implications for breast cancer prevention. Here, we establish that BRCA1 may exert its tumor-suppressor effects by conferring an antioxidant response in epithelial cells, thereby normally limiting excess hydrogen peroxide production. As such, both sporadic and familial cancers may use the same convergent biological mechanism(s): peroxide-induced oxidative stress and the "two-compartment tumor metabolism." Our data have obvious implications for the prevention of familial cancers in patients with inherited BRCA1 mutations, via prophylactic treatment with antioxidants, and possibly other familial cancers that use the same conserved peroxide-based mechanism(s).

Scientific). After blocking for 30 min in TBST (10mM TRIS-HCl pH 8.0, 150mM NaCl, 0.05% Tween-20) with 5% nonfat dry milk, membranes were incubated with the primary antibody for 1 h, washed and incubated for 30 min with horseradish peroxidase-conjugated secondary antibodies. The membranes were washed and incubated with enhanced chemiluminescence substrate (Thermo Scientific).

Measurement of hydrogen peroxide. PFBS-F is a cell-permeable, non-fluorescent molecule that, when taken up by cells, emits fluorescence in the presence of hydrogen peroxide, since it undergoes per-hydrolysis of the sulfonyl linkage. Fluorescence emission is hence a measure of intracellular hydrogen peroxide levels. The PFBS-F fluorescence method was used according to the manufacturer's instructions. Briefly, HCC1937 and HCC1937 + BRCA1 cells either in homotypic culture or with BJ-1 fibroblasts were cultured as described above. Cells were washed twice with PBS, incubated with 2.5 μM PFBS-F for 45 min at 37°C. Cells were then washed in PBS, harvested and re-suspended in 500 μL of PBS on ice. Cells were then analyzed by flow cytometry using a FITC signal detector to detect intensity of fluorescein emission. Data analysis was performed using FlowJo 8.8 software.

Apoptosis detection. Cell death was quantified by flow cytometry using propidium iodide (PI) and annexin-V-APC, as previously described with minor modifications.[63] Briefly, BJ1-GFP or BJ1-RFP cells were plated in 12-well plates, with either HCC1937 or HCC1937 + BRCA1 cells or MDA-MB-231 cells for 7 d, as described above. Ten mM NAC, or 2.5mM, 5 mM, 7.5 mM Tempol were added to the media 48 h or 24 h prior to harvesting, respectively; vehicle alone was added in the case of control conditions. NAC treatment was changed every 24 h for a total 48 h. Cells were collected by centrifugation and re-suspended in 500 μL of PBS. Then, the annexin-V-APC conjugate (4 μL) and PI (1 μL) was added and incubated in the dark at room temperature for 5 min. Cells were then analyzed by flow cytometry using a GFP or RFP signal detector (to detect BJ1-GFP or BJ1-RFP cells), a PE Texas Red signal detector for PI staining and an APC signal detector to detect annexin-V binding. GFP- or RFP-negative cells were the HCC1937 or HCC1937 + BRCA1 or MDA-MB-231 cells.

Immunocytochemistry. Cells were fixed after two to seven days of culture. Then, the ICC protocol was performed as previously described, with minor modifications.[32] Briefly, cells were fixed for 30 min at room temperature in 2% paraformaldehyde diluted in PBS, after which they were permeabilized with cold methanol at -20°C for 5 min. Cells were rinsed with PBS with 0.1 mM calcium chloride and 1 mM magnesium chloride (PBS/CM) and incubated with NH_4Cl in PBS to quench free aldehyde groups. After rinsing with PBS/CM, cells were blocked with IF buffer (PBS, 1% BSA, 0.1% Tween 20) for 1 h at room temperature. Primary antibodies were incubated in IF buffer for 1 h at room temperature. After washing with IF buffer (3x, 10 min each), cells were incubated for 30 min at room temperature with fluorochrome-conjugated anti-rabbit and anti-guinea pig secondary antibodies diluted in IF buffer. Finally, slides were washed at room temperature with IF buffer (3x, 10 min each), rinsed with PBS/CM, counter-stained with DAPI (10 μg/ml) in PBS and mounted with Prolong Gold anti-fade Reagent.

Confocal microscopy. Images were collected with a Zeiss LSM510 meta confocal system using a 405-nm diode excitation laser with a band pass filter of 420–480 nm, a 488 nm Argon excitation laser with a band pass filter of 505–550 nm and a 543-nm HeNe excitation laser with a 561–604-nm filter. Images were acquired with a 40x objective.

Immunohistochemistry. Paraffin-embedded sections of BRCA1-mutated human breast cancer samples were immunostained as previously described.[32,64] Briefly, sections were de-paraffinized, rehydrated and washed in PBS. Antigen retrieval was performed in 10 mM sodium citrate, pH 6.0 for 10 min using a pressure cooker. After blocking with 3% hydrogen peroxide for 10 min, sections were incubated with 10% goat serum for 1 h. Then, sections were incubated with primary antibodies overnight at 4°C. Antibody binding was detected using a biotinylated secondary (Vector Labs), followed by strepavidin-HRP (Dako). Immunoreactivity was revealed using 3, 3' diaminobenzidine.

Conclusions

In summary, our current work demonstrates that BRCA1 mutations in breast cancer drive oxidative stress and glycolytic transformation of the tumor stroma, with increased MCT4 and decreased Cav-1 expression. Antioxidant drugs can reverse this stromal transformation and induce epithelial cancer cell apoptosis. Hence, antioxidants need to be explored for the prophylaxis

and treatment of hereditary and basal-like breast cancers. Finally, our results provide new evidence that both sporadic and hereditary/familial breast cancers use the same conserved metabolic mechanism(s), reflecting the establishment of two-compartment tumor metabolism (**Fig. 11**). This has clear implications for breast cancer prevention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

U.E.M. was supported by a Young Investigator Award from the Margaret Q. Landenberger Research Foundation. Funds were also contributed by the Margaret Q. Landenberger Research Foundation (to M.P.L.).

This work was also supported, in part, by a Centre grant in Manchester from Breakthrough Breast Cancer in the UK and an Advanced ERC Grant from the European Research Council.

References

1. Foulkes WD. Inherited susceptibility to common cancers. N Engl J Med 2008; 359:2143-53; PMID:19005198; http://dx.doi.org/10.1056/NEJMra0802968.
2. Antoniou A, Pharoah PD, Narod S, Risch HA, Eyfjord JE, Hopper JL, et al. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. Am J Hum Genet 2003; 72:1117-30; PMID:12677558; http://dx.doi.org/10.1086/375033.
3. Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat Rev Cancer 2012; 12:68-78; PMID:22193408; http://dx.doi.org/10.1038/nrc3181.
4. Easton DF, Deffenbaugh AM, Pruss D, Frye C, Wenstrup RJ, Allen-Brady K, et al. A systematic genetic assessment of 1,433 sequence variants of unknown clinical significance in the BRCA1 and BRCA2 breast cancer-predisposition genes. Am J Hum Genet 2007; 81:873-83; PMID:17924331; http://dx.doi.org/10.1086/521032.
5. Yoshikawa K, Honda K, Inamoto T, Shinohara H, Yamauchi A, Suga K, et al. Reduction of BRCA1 protein expression in Japanese sporadic breast carcinomas and its frequent loss in BRCA1-associated cases. Clin Cancer Res 1999; 5:1249-61; PMID:10389907.
6. Rakha EA, El-Sheikh SE, Kandil MA, El-Sayed ME, Green AR, Ellis IO. Expression of BRCA1 protein in breast cancer and its prognostic significance. Hum Pathol 2008; 39:857-65; PMID:18400253; http://dx.doi.org/10.1016/j.humpath.2007.10.011.
7. Foulkes WD, Stefansson IM, Chappuis PO, Bégin LR, Goffin JR, Wong N, et al. Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. J Natl Cancer Inst 2003; 95:1482-5; PMID:14519755; http://dx.doi.org/10.1093/jnci/djg050.
8. Turner NC, Reis-Filho JS. Basal-like breast cancer and the BRCA1 phenotype. Oncogene 2006; 25:5846-53; PMID:16998499; http://dx.doi.org/10.1038/sj.onc.1209876.
9. Stefansson OA, Jonasson JG, Johannsson OT, Olafsdottir K, Steinarsdottir M, Valgeirsdottir S, et al. Genomic profiling of breast tumours in relation to BRCA abnormalities and phenotypes. Breast Cancer Res 2009; 11:R47; PMID:19589159; http://dx.doi.org/10.1186/bcr2334.
10. Turner NC, Reis-Filho JS, Russell AM, Springall RJ, Ryder K, Steele D, et al. BRCA1 dysfunction in sporadic basal-like breast cancer. Oncogene 2007; 26:2126-32; PMID:17016441; http://dx.doi.org/10.1038/sj.onc.1210014.
11. Guendel I, Carpio L, Pedati C, Schwartz A, Teal C, Kashanchi F, et al. Methylation of the tumor suppressor protein, BRCA1, influences its transcriptional cofactor function. PLoS One 2010; 5:e11379; PMID:20614009; http://dx.doi.org/10.1371/journal.pone.0011379.
12. Magdinier F, Ribieras S, Lenoir GM, Frappart L, Dante R. Down-regulation of BRCA1 in human sporadic breast cancer; analysis of DNA methylation patterns of the putative promoter region. Oncogene 1998; 17:3169-76; PMID:9872332; http://dx.doi.org/10.1038/sj.onc.1202248.
13. Rio PG, Maurizis JC, Peffault de Latour M, Bignon YJ, Bernard-Gallon DJ. Quantification of BRCA1 protein in sporadic breast carcinoma with or without loss of heterozygosity of the BRCA1 gene. Int J Cancer 1999; 80:823-6; PMID:10074913; http://dx.doi.org/10.1002/(SICI)1097-0215(19990315)80:6<823::AID-IJC5>3.0.CO;2-3.
14. Wilson CA, Ramos L, Villaseñor MR, Anders KH, Press MF, Clarke K, et al. Localization of human BRCA1 and its loss in high-grade, non-inherited breast carcinomas. Nat Genet 1999; 21:236-40; PMID:9988281; http://dx.doi.org/10.1038/6029.
15. Catteau A, Harris WH, Xu CF, Solomon E. Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics. Oncogene 1999; 18:1957-65; PMID:10208417; http://dx.doi.org/10.1038/sj.onc.1202509.
16. Esteller M, Silva JM, Dominguez G, Bonilla F, Matias-Guiu X, Lerma E, et al. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J Natl Cancer Inst 2000; 92:564-9; PMID:10749912; http://dx.doi.org/10.1093/jnci/92.7.564.
17. Stefansson OA, Jonasson JG, Olafsdottir K, Hilmarsdottir H, Olafsdottir G, Esteller M, et al. CpG island hypermethylation of BRCA1 and loss of pRb as co-occurring events in basal/triple-negative breast cancer. Epigenetics 2011; 6:638-49; PMID:21593597; http://dx.doi.org/10.4161/epi.6.5.15667.
18. Turner N, Tutt A, Ashworth A. Hallmarks of 'BRCAness' in sporadic cancers. Nat Rev Cancer 2004; 4:814-9; PMID:15510162; http://dx.doi.org/10.1038/nrc1457.
19. Metzger-Filho O, Tutt A, de Azambuja E, Saini KS, Viale G, Loi S, et al. Dissecting the heterogeneity of triple-negative breast cancer. J Clin Oncol 2012; 30:1879-87; PMID:22454417; http://dx.doi.org/10.1200/JCO.2011.38.2010.
20. Toullec A, Gerald D, Despouy G, Bourachot B, Cardon M, Lefort S, et al. Oxidative stress promotes myofibroblast differentiation and tumour spreading. EMBO Mol Med 2010; 2:211-30; PMID:20535745; http://dx.doi.org/10.1002/emmm.201000073.
21. Curtis CD, Thorngren DL, Nardulli AM. Immunohistochemical analysis of oxidative stress and DNA repair proteins in normal mammary and breast cancer tissues. BMC Cancer 2010; 10:9; PMID:20064251; http://dx.doi.org/10.1186/1471-2407-10-9.
22. Bae I, Fan S, Meng Q, Rih JK, Kim HJ, Kang HJ, et al. BRCA1 induces antioxidant gene expression and resistance to oxidative stress. Cancer Res 2004; 64:7893-909; PMID:15520196; http://dx.doi.org/10.1158/0008-5472.CAN-04-1119.
23. Saha T, Rih JK, Rosen EM. BRCA1 down-regulates cellular levels of reactive oxygen species. FEBS Lett 2009; 583:1535-43; PMID:19364506; http://dx.doi.org/10.1016/j.febslet.2009.04.005.
24. Esteve JM, Armengod ME, Knecht E. BRCA1 negatively regulates formation of autophagic vacuoles in MCF-7 breast cancer cells. Exp Cell Res 2010; 316:2618-29; PMID:20599945; http://dx.doi.org/10.1016/j.yexcr.2010.06.019.
25. Luini A, Aguilar M, Gatti G, Fasani R, Botteri E, Brito JA, et al. Metaplastic carcinoma of the breast, an unusual disease with worse prognosis: the experience of the European Institute of Oncology and review of the literature. Breast Cancer Res Treat 2007; 101:349-53; PMID:17009109; http://dx.doi.org/10.1007/s10549-006-9301-1.
26. Rakha EA, Reis-Filho JS, Ellis IO. Basal-like breast cancer: a critical review. J Clin Oncol 2008; 26:2568-81; PMID:18487574; http://dx.doi.org/10.1200/JCO.2007.13.1748.
27. Fulford LG, Easton DF, Reis-Filho JS, Sofronis A, Gillett CE, Lakhani SR, et al. Specific morphological features predictive for the basal phenotype in grade 3 invasive ductal carcinoma of breast. Histopathology 2006; 49:22-34; PMID:16842243; http://dx.doi.org/10.1111/j.1365-2559.2006.02453.x.
28. Storz P. Reactive oxygen species in tumor progression. Front Biosci 2005; 10:1881-96; PMID:15769673; http://dx.doi.org/10.2741/1667.
29. Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, et al. Cell transformation by the superoxide-generating oxidase Mox1. Nature 1999; 401:79-82; PMID:10485709; http://dx.doi.org/10.1038/43459.
30. Pelicano H, Lu W, Zhou Y, Zhang W, Chen Z, Hu Y, et al. Mitochondrial dysfunction and reactive oxygen species imbalance promote breast cancer cell motility through a CXCL14-mediated mechanism. Cancer Res 2009; 69:2375-83; PMID:19276362; http://dx.doi.org/10.1158/0008-5472.CAN-08-3359.
31. Martinez-Outschoorn UE, Lin Z, Trimmer C, Flomenberg N, Wang C, Pavlides S, et al. Cancer cells metabolically "fertilize" the tumor microenvironment with hydrogen peroxide, driving the Warburg effect: implications for PET imaging of human tumors. Cell Cycle 2011; 10:2504-20; PMID:21778829; http://dx.doi.org/10.4161/cc.10.15.16585.
32. Whitaker-Menezes D, Martinez-Outschoorn UE, Lin Z, Ertel A, Flomenberg N, Witkiewicz AK, et al. Evidence for a stromal-epithelial "lactate shuttle" in human tumors: MCT4 is a marker of oxidative stress in cancer-associated fibroblasts. Cell Cycle 2011; 10:1772-83; PMID:21558814; http://dx.doi.org/10.4161/cc.10.11.15659.
33. Martinez-Outschoorn UE, Balliet RM, Rivadeneira DB, Chiavarina B, Pavlides S, Wang C, et al. Oxidative stress in cancer associated fibroblasts drives tumor-stroma co-evolution: A new paradigm for understanding tumor metabolism, the field effect and genomic instability in cancer cells. Cell Cycle 2010; 9:3256-76; PMID:20814239; http://dx.doi.org/10.4161/cc.9.16.12553.
34. Migneco G, Whitaker-Menezes D, Chiavarina B, Castello-Cros R, Pavlides S, Pestell RG, et al. Glycolytic cancer associated fibroblasts promote breast cancer tumor growth, without a measurable increase in angiogenesis: evidence for stromal-epithelial metabolic coupling. Cell Cycle 2010; 9:2412-22; PMID:20562527; http://dx.doi.org/10.4161/cc.9.12.11989.

35. Witkiewicz AK, Whitaker-Menezes D, Dasgupta A, Philp NJ, Lin Z, Gandara R, et al. Using the "reverse Warburg effect" to identify high-risk breast cancer patients: stromal MCT4 predicts poor clinical outcome in triple-negative breast cancers. Cell Cycle 2012; 11:1108-17; PMID:22313602; http://dx.doi.org/10.4161/cc.11.6.19530.
36. Witkiewicz AK, Dasgupta A, Sammons S, Er O, Potoczek MB, Guiles F, et al. Loss of stromal caveolin-1 expression predicts poor clinical outcome in triple negative and basal-like breast cancers. Cancer Biol Ther 2010; 10:135-43; PMID:20431349; http://dx.doi.org/10.4161/cbt.10.2.11983.
37. Witkiewicz AK, Dasgupta A, Sotgia F, Mercier I, Pestell RG, Sabel M, et al. An absence of stromal caveolin-1 expression predicts early tumor recurrence and poor clinical outcome in human breast cancers. Am J Pathol 2009; 174:2023-34; PMID:19411448; http://dx.doi.org/10.2353/ajpath.2009.080873.
38. Martinez-Outschoorn UE, Pavlides S, Whitaker-Menezes D, Daumer KM, Milliman JN, Chiavarina B, et al. Tumor cells induce the cancer associated fibroblast phenotype via caveolin-1 degradation: implications for breast cancer and DCIS therapy with autophagy inhibitors. Cell Cycle 2010; 9:2423-33; PMID:20562526; http://dx.doi.org/10.4161/cc.9.12.12048.
39. Martinez-Outschoorn UE, Trimmer C, Lin Z, Whitaker-Menezes D, Chiavarina B, Zhou J, et al. Autophagy in cancer associated fibroblasts promotes tumor cell survival: Role of hypoxia, HIF1 induction and NFκB activation in the tumor stromal microenvironment. Cell Cycle 2010; 9:3515-33; PMID:20855962; http://dx.doi.org/10.4161/cc.9.17.12928.
40. Guido C, Whitaker-Menezes D, Capparelli C, Balliet R, Lin Z, Pestell RG, et al. Metabolic reprogramming of cancer-associated fibroblasts by TGF-β drives tumor growth: connecting TGF-β signaling with "Warburg-like" cancer metabolism and L-lactate production. Cell Cycle 2012; 11:3019-35; PMID:22874531; http://dx.doi.org/10.4161/cc.21384.
41. Tomlinson GE, Chen TT, Stastny VA, Virmani AK, Spillman MA, Tonk V, et al. Characterization of a breast cancer cell line derived from a germ-line BRCA1 mutation carrier. Cancer Res 1998; 58:3237-42; PMID:9699648.
42. Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. J Biol Chem 2006; 281:9030-7; PMID:16452478; http://dx.doi.org/10.1074/jbc.M511397200.
43. Chiavarina B, Whitaker-Menezes D, Migneco G, Martinez-Outschoorn UE, Pavlides S, Howell A, et al. HIF1-alpha functions as a tumor promoter in cancer associated fibroblasts, and as a tumor suppressor in breast cancer cells: Autophagy drives compartment-specific oncogenesis. Cell Cycle 2010; 9:3534-51; PMID:20864819; http://dx.doi.org/10.4161/cc.9.17.12908.
44. Chiavarina B, Whitaker-Menezes D, Martinez-Outschoorn UE, Witkiewicz AK, Birbe RC, Howell A, et al. Pyruvate kinase expression (PKM1 and PKM2) in cancer-associated fibroblasts drives stromal nutrient production and tumor growth. Cancer Biol Ther 2011; 12: In press; PMID:22236875.
45. Sotgia F, Martinez-Outschoorn UE, Howell A, Pestell RG, Pavlides S, Lisanti MP. Caveolin-1 and cancer metabolism in the tumor microenvironment: markers, models, and mechanisms. Annu Rev Pathol 2012; 7:423-67; PMID:22077552; http://dx.doi.org/10.1146/annurev-pathol-011811-120856.
46. Simpkins SA, Hanby AM, Holliday DL, Speirs V. Clinical and functional significance of loss of caveolin-1 expression in breast cancer-associated fibroblasts. J Pathol 2012; 227:490-8; PMID:22488553; http://dx.doi.org/10.1002/path.4034.
47. Wu KN, Queenan M, Brody JR, Potoczek M, Sotgia F, Lisanti MP, et al. Loss of stromal caveolin-1 expression in malignant melanoma metastases predicts poor survival. Cell Cycle 2011; 10:4250-5; PMID:22134245; http://dx.doi.org/10.4161/cc.10.24.18551.
48. Di Vizio D, Morello M, Sotgia F, Pestell RG, Freeman MR, Lisanti MP. An absence of stromal caveolin-1 is associated with advanced prostate cancer, metastatic disease and epithelial Akt activation. Cell Cycle 2009; 8:2420-4; PMID:19556867; http://dx.doi.org/10.4161/cc.8.15.9116.
49. Tilanus-Linthorst M, Verhoog L, Obdeijn IM, Bartels K, Menke-Pluymers M, Eggermont A, et al. A BRCA1/2 mutation, high breast density and prominent pushing margins of a tumor independently contribute to a frequent false-negative mammography. Int J Cancer 2002; 102:91-5; PMID:12353239; http://dx.doi.org/10.1002/ijc.10666.
50. Metcalfe K, Lynch HT, Ghadirian P, Tung N, Olivotto I, Warner E, et al. Contralateral breast cancer in BRCA1 and BRCA2 mutation carriers. J Clin Oncol 2004; 22:2328-35; PMID:15197194; http://dx.doi.org/10.1200/JCO.2004.04.033.
51. Haffty BG, Harrold E, Khan AJ, Pathare P, Smith TE, Turner BC, et al. Outcome of conservatively managed early-onset breast cancer by BRCA1/2 status. Lancet 2002; 359:1471-7; PMID:11988246; http://dx.doi.org/10.1016/S0140-6736(02)08434-9.
52. Liebens FP, Carly B, Pastijn A, Rozenberg S. Management of BRCA1/2 associated breast cancer: a systematic qualitative review of the state of knowledge in 2006. Eur J Cancer 2007; 43:238-57; PMID:17095205; http://dx.doi.org/10.1016/j.ejca.2006.07.019.
53. Garcia-Etienne CA, Barile M, Gentilini OD, Botteri E, Rotmensz N, Sagona A, et al. Breast-conserving surgery in BRCA1/2 mutation carriers: are we approaching an answer? Ann Surg Oncol 2009; 16:3380-7; PMID:19649554; http://dx.doi.org/10.1245/s10434-009-0638-7.
54. Schrag D, Kuntz KM, Garber JE, Weeks JC. Life expectancy gains from cancer prevention strategies for women with breast cancer and BRCA1 or BRCA2 mutations. JAMA 2000; 283:617-24; PMID:10665701; http://dx.doi.org/10.1001/jama.283.5.617.
55. Anderson K, Jacobson JS, Heitjan DF, Zivin JG, Hershman D, Neugut AI, et al. Cost-effectiveness of preventive strategies for women with a BRCA1 or a BRCA2 mutation. Ann Intern Med 2006; 144:397-406; PMID:16549852.
56. Kauff ND, Domchek SM, Friebel TM, Robson ME, Lee J, Garber JE, et al. Risk-reducing salpingo-oophorectomy for the prevention of BRCA1- and BRCA2-associated breast and gynecologic cancer: a multicenter, prospective study. J Clin Oncol 2008; 26:1331-7; PMID:18268356; http://dx.doi.org/10.1200/JCO.2007.13.9626.
57. Kang HJ, Hong YB, Kim HJ, Wang A, Bae I. Bioactive food components prevent carcinogenic stress via Nrf2 activation in BRCA1 deficient breast epithelial cells. Toxicol Lett 2012; 209:154-60; PMID:22192953; http://dx.doi.org/10.1016/j.toxlet.2011.12.002.
58. Kowalska E, Narod SA, Huzarski T, Zajaczek S, Huzarska J, Gorski B, et al. Increased rates of chromosome breakage in BRCA1 carriers are normalized by oral selenium supplementation. Cancer Epidemiol Biomarkers Prev 2005; 14:1302-6; PMID:15894690; http://dx.doi.org/10.1158/1055-9965.EPI-03-0448.
59. Dziaman T, Huzarski T, Gackowski D, Rozalski R, Siomek A, Szpila A, et al. Selenium supplementation reduced oxidative DNA damage in adnexectomized BRCA1 mutations carriers. Cancer Epidemiol Biomarkers Prev 2009; 18:2923-8; PMID:19843683; http://dx.doi.org/10.1158/1055-9965.EPI-09-0529.
60. Livasy CA, Karaca G, Nanda R, Tretiakova MS, Olopade OI, Moore DT, et al. Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. Mod Pathol 2006; 19:264-71; PMID:16341146; http://dx.doi.org/10.1038/modpathol.3800528.
61. Gazdar AF, Kurvari V, Virmani A, Gollahon L, Sakaguchi M, Westerfield M, et al. Characterization of paired tumor and non-tumor cell lines established from patients with breast cancer. Int J Cancer 1998; 78:766-74; PMID:9833771; http://dx.doi.org/10.1002/(SICI)1097-0215(19981209)78:6<766::AID-IJC15>3.0.CO;2-L.
62. Gallagher SM, Castorino JJ, Wang D, Philp NJ. Monocarboxylate transporter 4 regulates maturation and trafficking of CD147 to the plasma membrane in the metastatic breast cancer cell line MDA-MB-231. Cancer Res 2007; 67:4182-9; PMID:17483329; http://dx.doi.org/10.1158/0008-5472.CAN-06-3184.
63. Martinez-Outschoorn UE, Goldberg A, Lin Z, Ko YH, Flomenberg N, Wang C, et al. Anti-estrogen resistance in breast cancer is induced by the tumor microenvironment and can be overcome by inhibiting mitochondrial function in epithelial cancer cells. Cancer Biol Ther 2011; 12:924-38; PMID:22041887; http://dx.doi.org/10.4161/cbt.12.10.17780.
64. Whitaker-Menezes D, Martinez-Outschoorn UE, Flomenberg N, Birbe RC, Witkiewicz AK, Howell A, et al. Hyperactivation of oxidative mitochondrial metabolism in epithelial cancer cells in situ: visualizing the therapeutic effects of metformin in tumor tissue. Cell Cycle 2011; 10:4047-64; PMID:22134189; http://dx.doi.org/10.4161/cc.10.23.18151.