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Bio-electric Field Enhancement: the Influence on Hyaluronan Mediated Motility Receptors in Human Breast Carcinoma

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Abstract: Mechanisms that regulate cancer cell metastasis are often intricately linked to mechanisms that control cell migration in wound repair. Hyaluronan Mediated Motility Receptor *(HMMR)* encodes a receptor for hyaluronan-mediated motility (RHAMM), a non-integral cell surface hyaluronan receptor and intracellular protein that promotes mitotic spindle formation and cell motility. RHAMM has been found to have increased expression in both cancers and wounds, and when cancers show increased RHAMM expression poor outcomes have occurred. Therefore, RHAMM has been shown to contribute to both natural healing mechanisms and cancer cell pathology. RHAMM is expressed in breast tissue and forms a polarity normalizing complex with *BRCA1.* Mutations of *BRCA1* have been associated with a loss of apicobasal cell polarity along with a subsequent increased expression of RHAMM. Here we show how a human breast carcinoma cell line was maintained in media prepared with a dilute saline solution that had been exposed to a dielectrophoretic (DEP) electromagnetic field (EMF) generated by 3 amperes of direct current (dc) to a device housing an array of conductive rings. This Bio-electric Field Enhancement (BEFE) device has been available commercially for use in baths/footbaths since 1996 and consumers claim that it provides health benefits ranging from lowering blood pressure to faster wound healing. Our studies showed a significant inhibition of growth of human breast carcinoma MDA-MB-231 cells when they were maintained in media prepared with dc-DEP EMF force treated dilute saline while no growth inhibition occurred when the same cells were maintained in identically prepared but untreated media. Importantly, no growth inhibition was observed in human epithelial MCF-10A cells when grown in either treated or untreated media. Also, mitotic spindle formation was inhibited in the human breast carcinoma when they were grown in dc-DEP EMF force treated media. To determine if any gene expression changes contributed to the selective growth inhibition and absent mitotic spindle formation in the human breast carcinoma grown in the treated media, we employed microarray analysis and found that there was large-scale transcriptional reprogramming of the tumor cells grown in the treated media with over 1,000 genes up- or down-regulated over 2-fold, whereas the non-cancerous MCF-10A cells showed relatively modest changes in gene expression. Of the genes affected in the MDA-MB-231 cells, the significance of down-regulation of *HMMR* is discussed. The ability to enhance cell polarity through the application of this dc-DEP EMF force may offer another way to stabilize *HMMR* and differentially modulate its expression in cancerous and noncancerous cells. [*Discovery Medicine* 23(127):n-n, 2017]

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Introduction

In 2016 there were estimated 246,660 new cases of invasive breast cancer and 61,000 new cases of non-invasive breast cancer diagnosed in women in the United States (ASCO, 2016). About 5-10% of breast cancers can be linked to genetic mutations that are inherited (Greenberg, 2006). The most common inherited genetic mutations occur in the *BRCA1/2* genes (Greenberg, 2006). Women with the *BRCA1* mutation

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have a 55-65% lifetime risk of developing breast cancer while women with the *BRCA2* mutation have a 45% increased risk (Chen and Parmigiani, 2007). Breast cancer tends to develop more often in younger women who test positive for these inherited mutations. An increased risk of ovarian and other cancers in both men and women is also associated with these inherited mutations (Lynch *et al*., 2009).

Hyaluronan-mediated motility receptor (*HMMR*), also known as RHAMM (Receptor for Hyaluronan Mediated Motility), is a non-integral cell surface hyaluronan receptor (CD168) and intracellular protein that is encoded by the *HMMR* gene and promotes cell motility in culture (Shigeishi *et al*., 2014; Hamilton *et al*., 2007). This protein is located in the cytoskeleton and centrosome and has been linked to modulation of growth factor receptors and regulation of cell signaling pathways and mitotic spindle assembly (Maxwell *et al*., 2008). RHAMM has been known to play a significant role in tumorigenesis, genomic instability, and cancer progression. RHAMM is expressed in breast tissue and forms a complex with *BRCA1* and other proteins (*BRCA1-AURKA-RHAMM-TPX2-TUBG1*) that are known to regulate mammary cell polarization that is necessary for mammary stem cell fate. The loss of tissue polarity, due to *BRCA1* mutation or other possible factors that change the cell microenvironment, is an early hallmark of breast cancer (Maxwell, 2011; Blanco *et al*., 2015) (Figure 3A). Also, terminal differentiation of mammary epithelial cells is thought to be mediated by both *BRCA1* activation and RHAMM degradation (Maxwell, 2011; Blanco *et al*., 2015). Interestingly, an increased cell motility/metastasis is seen through a number of different pathways with an increased RHAMM (Maxwell, 2011; Blanco *et al*., 2015). RHAMM is unconventionally exported to the cell surface in response to stimuli such as wounding and cytokine release. While this may be an innate compensatory response that is designed to facilitate wound healing and the necessary cell migration that is needed for healing and repair, it can also have devastating consequences when this response leads to cell migration/metastasis of a cancerous cell (Tolg *et al*., 2014; Purnell and Skrinjar, 2016). Triple negative and HER2 subtype breast cancers have been found to have increased expression of RHAMM and patients with this increased expression have often suffered increased metastasis and ultimately poor outcomes and decreased survival (Edward *et al*., 2005).

Here we will investigate how a direct current (dc) dielectrophoretic (DEF) electromagnetic field (EMF) force that is generated by the Bio-electric Field Enhancement (BEFE) Unit appears to enhance cell polarity through the effect of bio-chloride ($_{b}Cl^{-}$) on diamagnetic anisotropy (Purnell and Skrinjar, 2016). The BEFE unit has been used for two decades across the globe as a footbath/bath with many interesting anecdotal reports of numerous health benefits (Purnell and Skrinjar, 2016). These experiments investigate how exposing a dilute saline solution to the dc-DEF EMF force generated by the BEFE and then using this saline to reconstitute growth media of MDA-MB-231 and MCF-10A cells affects cell growth over time and ultimately gene expression *in vitro*. These findings of dc-DEP EMF force driven enhanced cell polarity were found to show a significant growth inhibition in the human breast carcinoma without showing a growth inhibition in the human breast epithelial cells (Purnell and Skrinjar, 2016). Apicobasal polarity is a unique finding in epithelial cells since it refers to the polarity interplay between the apical membrane and the basolateral membrane, separated by tight junctions (Khursheed and Bashyam, 2014). This polarity is an important part of the cytoskeletal reorganization, Wnt signaling, TGFB, integrin-mediated signaling, and differentiation (Royer and Lu, 2011). Since *BRCA1* mutations are thought to lead to loss of cell polarity and ultimately increased *HMMR* (increased migration/metastasis) expression (Furuta *et al*., 2005), the ability to enhance cell polarity with this dc-DEP EMF force generated by the BEFE device could offer another way to stabilize cell polarity in both the absence of an intact *BRCA1* influence as well as other adverse conditions of the cell microenvironment. This polarity stabilization could not only decrease the risk of developing cancer and subsequent metastasis with these inherited genetic mutations, but also could possibly help to reduce these findings in other genetically driven aggressive cancers (Lobikin *et al*., 2012).

Experimental Methods and Design

Cells and cell culture media

Human MDA-MB-231 triple-negative breast carcinoma and human MCF-10A breast epithelial cells were obtained from the American Type Culture Collection (Manassas, VA). The MDA-MB-231 cells were maintained in high glucose Dulbecco's Modified Eagles Medium (DMEM, Lonza) containing 10% fetal bovine serum (FBS; Atlanta Biologicals). MCF-10A cells were maintained in DMEM/F12 (Invitrogen) supplemented with 20 ng/ml epidermal growth factor (PeproTech), 0.5 mg/ml hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), 10 μg/ml insulin (Sigma-Aldrich), and 5% horse serum (Invitrogen).

To prepare treated and control DMEM, 10x DMEM (Sigma-Aldrich) was diluted 9:1 with a hypotonic saline solution that had been treated for 30 minutes at 3 amperes of dc with the dc-DEP-EMF device, or with an aliquot of the same solution prior to treatment with the device. The hypotonic saline solution consisted of 3 mM NaCl prepared using laboratory-grade deionized water and molecular biology-grade NaCl (Promega). Complete treated and control DMEM was supplemented with 0.004 gm/L folic acid (Sigma-Aldrich), 4,000 mg/L glucose (Sigma-Aldrich), 0.584 gm/L glutamine (Sigma-Aldrich) and 3.7 gm/L sodium bicarbonate (Biowhittaker) and filtered through a 0.45 micron pore size bottle top filter (Corning). Fetal bovine serum was then added to 10% final concentration.

To prepare treated and control media for growth of the MCF-10A cells, F-12 nutrient mix powder (Life technologies) was re-suspended in either dc-DEP-EMF-treated saline, or with an aliquot of the same solution prior to treatment with the device. The F-12 media was then mixed 1:1 with DMEM prepared as described above with either dc-DEP-EMF-treated or control saline and then the DMEM/F-12 was supplemented with EGF, cholera toxin and insulin as described above and then filter sterilized as described above. Horse serum was then added to 5% final concentration. Each of the two cell lines was cultured in the growth media that has been shown to facilitate their optimal growth and is recommended by ATCC.

Cell growth studies

We cultured the treated groups of MDA-MB231 cells in the DMEM-10 with media that was reconstituted with a hypotonic saline solution that had been treated with the dc-DEP force EMF for 30 minutes and the control groups were cultured in media that was reconstituted with the same hypotonic saline solution prior to treatment with the BEFE system. The treated group of MCF-10A cells was cultured in the DMEM/F12-5 media that had been reconstituted with a hypotonic saline solution that had been treated with the dc-DEP force EMF for 30 minutes and the control group was cultured in media that was reconstituted with a hypotonic saline solution that had not been treated with the dc-DEP force EMF. On day one, aliquots of 10,000 cells were plated in three 6-well plates for each of the two groups for each of the two cell lines. They were plated in their standard (non-EMF treated) DMEM-10 or DMEM/F12-5 media on day 1. On day 2, the treated (n=21) and control (n=21) media for each of the two cell lines were made and the original standard media was replaced in each of the wells with the newly prepared treated and control media. On days 3 through 7, media was prepared and changed daily and wells from the control group and treated group of each cell line were trypsinized, removed from 3 wells of each group and counted and cell size of each sample was measured using a Scepter cell counter (EMD Millipore). When normality is not met with the data, the recommended non-parametric test to replace the student's t-test is the Mann-Whitney U analysis.

Mitotic index by tubulin staining

Replicate 60 mm dishes of MDA-MB231 cells (6 plates of each control and treated) were plated in standard DMEM-10 media. On day 2 the media in the control and treated groups were replaced and the cells were allowed to grow for 1 day. On day 3 the cells were washed with PBS and fixed with paraformaldehyde. The cells were then stained with DAPI (4′,6-diamidino-2-phenylindole). Then an anti-tubulin stain was added prior to staining the cytoskeleton/microtubules with rhodamine-conjugated secondary antibody (red). Cells were then examined by fluorescent microscopy and the percentages of cells seen in the different stages of mitosis were calculated to achieve a mitotic index.

Microarray analysis

Replicate 60 mm dishes of either MDA-MB-231 or MCF-10A (5 plates each for growth in treated and control media) were plated in DMEM-10 and the next day the media was replaced with either treated or control media which was replaced daily with freshly prepared treated or control media for the next two days. On day 4 post-plating (day 3 post-treatment) the cells were removed with trypsin, counted and 3×10^6 cells from each plate were collected by centrifugation and total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen). RNA concentration was determined and RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies) and all RNA integrity number (RIN) values were $\geq$10. The RNAs from the five biologic replicates from each group were combined and cDNA was generated using Ambion WT amplification kit (ThermoFisher Scientific) according to the manufacturer's instructions. The samples were subsequently fragmented and labeled using the Affymetrix WT Terminal Labeling kit and then hybridized, together with the probe array controls, onto the Human Genome U133 Plus 2.0 GeneChip Array (Affymetrix). The array was washed and stained using an Affymetrix Fluidics Station 450, scanned on an Affymetrix GCS3000 7G scanner, and the data were normalized by Robust Multichip Averaging (RMA) using the Affymetrix

expression console in order to transform all the arrays to have a common distribution of intensities by removing technical variation from noisy data before analysis. To quantile-normalize two or more distributions to each other, both treated and control groups were set to the average (arithmetical mean) of both distributions. Therefore, the highest value in all cases becomes the mean of the highest values, and the second highest value becomes the mean of the second highest values.

Quantitative RT-PCR

Hyaluronan-mediated motility receptor (*HMMR*) showed a significant difference in expression between the treated and control MDA-MB-231 groups in the microarray analyses and when validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Ribosomal protein S19 (*RIBOPROTS19*) was used for normalization. The primers were designed using the Universal Probe Library Probe Finder assay design software (Roche) and sequences are available upon request. RNAs from the 5 biological replicates were reverse-transcribed individually using the Transcriptor First Strand cDNA Synthesis Kit (Roche) to generate cDNAs according to the manufacturer's protocol. Ten-fold serial dilutions (10^{-1}-10^{-4}) of each of the cDNAs were then mixed with the appropriate universal library probe (Universal ProbeLibrary; Roche), sense and anti-sense primers, and reaction buffer into 96-well plates. The polymerase was activated by incubation at 95°C for 5 minutes after a 45-cycle amplification consisting of denaturation at 95°C for 15 seconds, annealing at 60°C for 1 minute and elongation at 72°C for 5 minutes.

Results

Cell growth and mitotic spindle

Our initial experiments showed significant growth inhibition of the human breast carcinoma when grown in the dc-DEP EMF force treated media when compared to the control group which showed no growth inhibition (Figure 1A). Alternatively, the human breast epithelial cells showed no growth inhibition between the control and treated groups (Figure 1B). After 1 day of growth in the treated media, the tubulin staining of the human breast carcinoma showed a significant difference in the mitotic spindle formation between the control and treated groups. The control group showed approximately 20% of the cells undergoing mitotic spindle formation while 0% of the cells undergoing mitotic spindle formation were noted in the treated groups (Figure 2).

Microarray and RT-qPCR

Microarray analysis of the control versus treated groups of the human breast carcinoma identified 1,165 genes that were up-regulated over 2-fold and 872 genes that

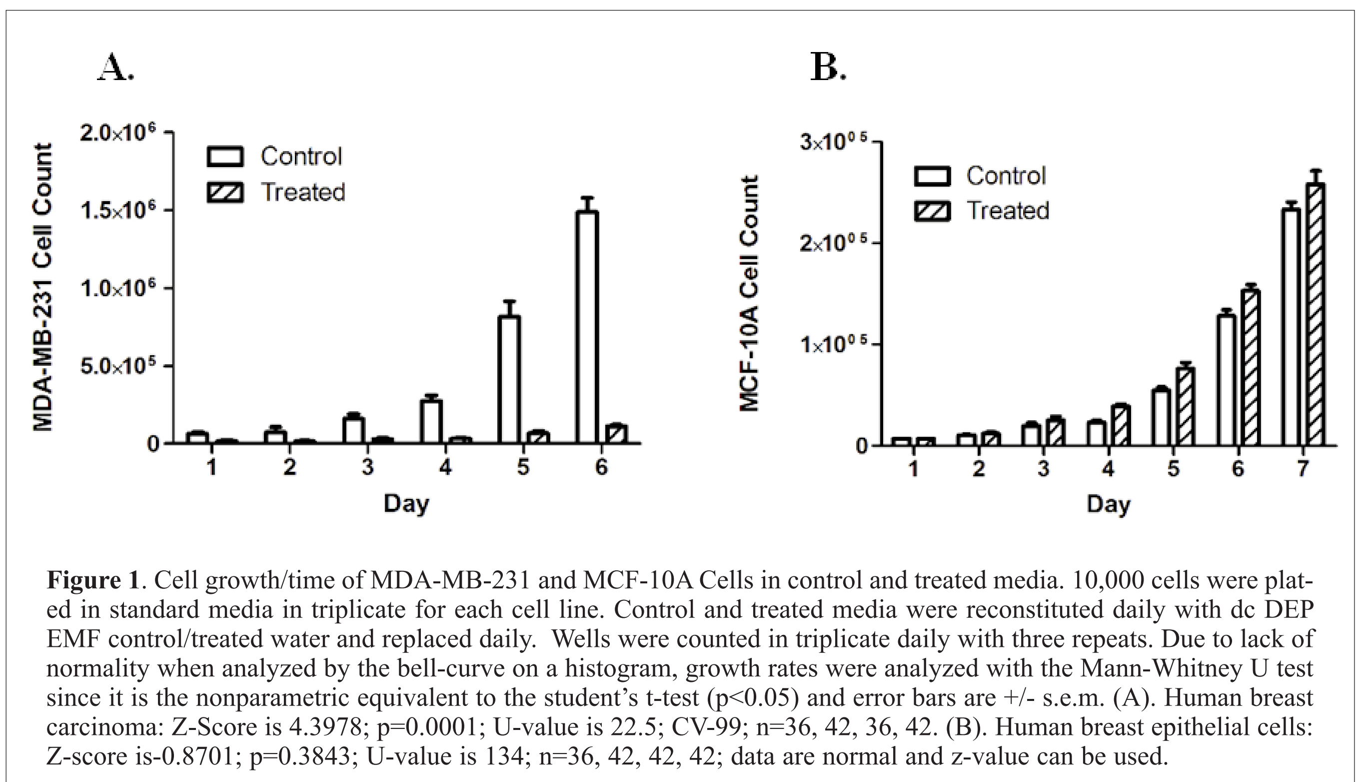

Figure 1. Cell growth/time of MDA-MB-231 and MCF-10A Cells in control and treated media. 10,000 cells were plated in standard media in triplicate for each cell line. Control and treated media were reconstituted daily with dc DEP EMF control/treated water and replaced daily. Wells were counted in triplicate daily with three repeats. Due to lack of normality when analyzed by the bell-curve on a histogram, growth rates were analyzed with the Mann-Whitney U test since it is the nonparametric equivalent to the student's t-test ($p<0.05$) and error bars are +/- s.e.m. (A). Human breast carcinoma: Z-Score is 4.3978; p=0.0001; U-value is 22.5; CV-99; n=36, 42, 36, 42. (B). Human breast epithelial cells: Z-score is-0.8701; p=0.3843; U-value is 134; n=36, 42, 42, 42; data are normal and z-value can be used.

were down-regulated over 2-fold in the treated groups grown in the media that was reconstituted with the dc-DEP force EMF-treated hypotonic saline solution. For comparison, 431 transcripts showed over 2-fold changes in the dc-DEP force EMF-treated versus non-treated human epithelial cells. One of the genes that were noted to be significantly changed between the treated and control groups of the human breast carcinoma in the microarray analyses was *HMMR*. *HMMR* showed significant down-regulation by RT-qPCR via unpaired t-tests (Table 1) along with a 16-fold decrease in expression by analysis of the delta-delta CT method in the treated group. The delta-delta CT method has been used extensively as a relative quantification method for quantitative real-time polymerase chain reaction data analysis. The relative quantification determines gene expression relative to a reference sample (housekeeping gene). While there was a slight decrease in gene expression on microarray analysis, there was no significant change in gene expression by RT-qPCR between the treated versus control groups of the MCF-10A epithelial cells when analyzed with unpaired t-tests (Table 1).

Discussion

Recent studies suggest *HMMR* is an oncogene that can enhance tumor invasion by multiple functional mechanisms that occur in select subcellular locations (Tilghman *et al*., 2014). These locations include the intracellular regulation of mitotic-spindle assembly and extracellular activation of CD44 motogenic/invasive functions as well as Ras signaling pathways (Maxwell *et al*., 2008). Extracellular *HMMR* forms a complex with CD44, which then combines with hyaluronan and activates intracellular signaling pathways such as extracellular signal-regulated kinase (ERK) that regulate tumor cell proliferation and migration (Tilghman *et al*., 2014). RHAMM, a protein encoded by *HMMR,* is not highly expressed in normal tissues but has been found to be overexpressed in many advanced human cancers (Edward *et al*., 2005). Also, RHAMM is a mitotic-spindle/hyaluronan binding cytoplasmic and nuclear protein (Nickel, 2005) that shows interaction with interphase microtubules, centrosomes, and the mitotic spindle formation, revealing its multiple functions across multiple cell compartments (Assmann *et al*., 1999). Spindle forming functions of RHAMM have been shown to be inhibited by the breast/ovarian tumor suppressor gene *BRCA1*. *BRCA1* has also been shown to participate in the maintenance of apicobasal membrane polarity. Therefore, when a *BRCA1* mutation occurs, the loss of cell polarity and the lack of silencing of *HMMR* from the *BRCA1* gene could be a contributing

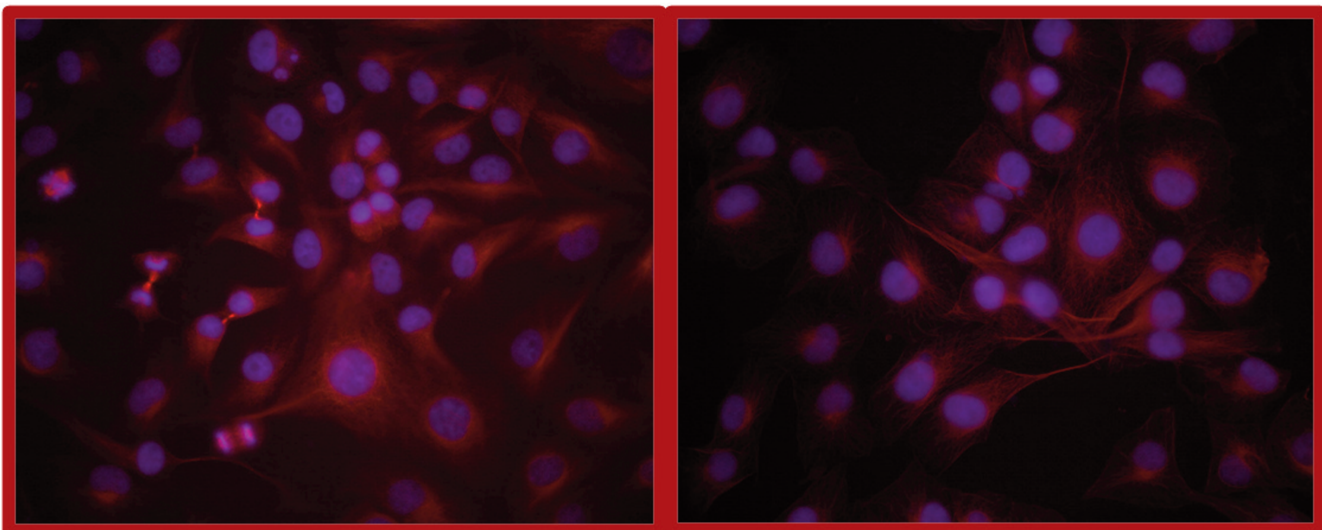

Figure 2. Mitosis/mitotic spindle formation of MDA-MB231 cells in control (left) and treated (right) after 24 hours of growth in control and treated media with 100 X magnification. Cells were fixed and stained with DAPI to visualize the nuclei and then with anti-tubulin antibody followed by a rhodamine-conjugated secondary antibody for fluorescent microscopy analyses. Note the mitotic spindle formation that is occurring in the control human breast carcinoma (left) and note the lack of mitotic spindle formation with down-regulation of HMMR in the treated human breast carcinoma (right). Upon analysis, there were approximately 20% of the control cells undergoing some stage of mitosis (mitotic index) while there were 0% of the treated cells undergoing mitosis (n=36, n=12, n=36).

factor to tumor development, progression, and metastasis (Figure 3B). Also, in the absence of a *BRCA1* mutation, the overexpression of RHAMM in cancers correlates with increased metastasis and poor outcomes suggesting that this protein may be highly influential in both inherited and non-inherited forms of breast cancer (Auvinen *et al*., 2000; Blanco *et al*., 2015). Our experiments using the dc-DEP-EMF application with the MDA-MB231 cells show a significant growth inhibition (Figure 1) and changes in mitotic spindle formation (Figure 2) when these cells are grown in the dc-DEP-EMF treated growth media compared to controls. There have also been changes in chloride ion channel expression and cell polarity with this dc-DEP EMF force application from the BEFE unit (Purnell and Skrinjar, 2016). The significant down-regulation of *HMMR* in our RT-qPCR analysis when coupled with the decreased cell growth/mitosis and decreased mitotic spindle formation in the treated versus control human breast carcinoma may suggest that a decreased cell motility/migration/metastasis could occur when the cancerous cells are exposed to the dc-DEP EMF force. Interestingly, there was no decreased growth/mitosis noted in the human breast epithelial cells and *HMMR* showed no significant change in the human breast epithelial cells in our microarray analyses. RHAMM has also been shown to initiate multiple motogenic signaling pathways such as Ras, pp60-c-SRC, ERK1, and ERK2 (Wang *et al*., 1998). It has also been shown to perform motogenic and invasive functions that are similar to, and may actually enhance, CD44 (Hamilton *et al*., 2007). While RHAMM has been less studied than CD44 with regard to cancer metastasis, it is likely just as important. Once a metastatic lesion is formed, increased RHAMM will also partner with CD44 to promote angiogenesis (Savani *et al*., 2001; Hamilton *et al*., 2007).

We know that cell polarity differs in cancerous, injured, and proliferating cells where transmembrane potential is ~<-30 mV, while noncancerous cells have a resting potential of ~>-70 mV (Zhou and Uesaka, 2006; Yang and Brackenbury, 2013; Levin, 2014; Lobikin *et al*., 2012). Since this dc-DEP-EMF produced by the BEFE has been shown to hyperpolarize both human breast carcinoma and human breast epithelial cells *in vitro* (Purnell and Skrinjar, 2016), further experiments should be conducted to see if this applied dc-DEP-EMF could in fact offer the polarization stability that is lacking with both the inherited *BRCA1* mutations and other non-inherited cancers. A depolarization of the human mammary tissue, as is often seen in wounds and inflammatory microenvironments, could also occur in the absence of the *BRCA1* mutation and possibly activate *HMMR* expression and other cell migratory pathways (Furuta, 2005; Purnell and Skrinjar, 2016). Initiation of cell migration pathways occurs when cells are damaged or depolarized and these same mechanisms that spark wound healing may initiate tumor cell metastasis. We propose that this dc DEP EMF force that is generated by the BEFE device may in fact differentially *enhance* wound healing and cell migration in noncancerous cells while *inhibiting* cell migration/metastasis in cancerous cells (Purnell and Skrinjar, 2016a; Purnell and Skrinjar, 2016b). The MCF-10A cells do not show a significant change in *HMMR* expression by RT-qPCR. This could be due to the fact that the expression in these cells is not abnormally elevated due to a pre-existing depolarized (stressed) state prior to exposure to the dc-DEP-EMF treated media. Therefore, control of *HMMR* expression through the application of this dc-DEP EMF force could be a possible future application to the tumor microenvironment that allows for control of tumor development, progression, and metastasis by maintaining cell polarity in the presence of *BRCA1* mutations and other depolarized cell states (Figure 3C).

Conclusion

The dc-DEF EMF force leads to a significantly decreased expression in *HMMR* in human breast carcinoma and could offer a novel approach to developing an understanding of the relationship between the tumor

Table 1. Unpaired T-Tests of RT-qPCR for HMMR Expression Between Treated and Control Groups of MDA-MB231 Cells and MCF-10A Cells.

Gene	Unpaired t-Tests			Mean/Control	Mean/Treated
	df	ts	p		
MDA-MB231 *HMMR*	8	6.3889	0.0002	6.7446	10.3200
MCF-10A *HMMR*	8	0.0580	0.9551	12.6670	12.6770

df, degrees of freedom; ts, test statistic; p, p-value.

microenvironment and tumor initiation and progression. The mechanisms that link cell migration/wound healing and cell migration/cancer metastasis pose an example of how challenging the development of side effects free cancer treatments can be (Schafer and Werner, 2008). A potential key to unlocking the mystery of how to stop cancer metastasis without affecting the healing in normal cell migration and repair can be found in the ability to control cell polarity and *HMMR* expression under the influence of $_bCl^-$ on cell membrane diamagnetic anisotropy that is generated by this dc-DEP EMF force (Purnell and Skrinjar, 2016) (Figure 3C). The dc-DEP-EMF shows the possibility of offering changes in epigenetic expression in the presence of both inherited genetic mutations (*BRCA1*) and non-inherited mutations that are involved in tumor progression.

Studies have shown that women who test positive for *BRCA1* mutations and are treated for Stage I or II breast cancer with bilateral mastectomy are less likely to die from breast cancer than women who are treated with unilateral mastectomy (Metcalf *et al*., 2014). Also, the rates of contralateral prophylactic mastectomy (CPM) in women with no inherited mutations and unilateral breast cancer are increasing despite the fact that the majority of women achieve no oncologic benefit from the CPM (Fisher, 2016). There are many reasons that women chose CPM, and the predominant theme for many is often peace of mind. RHAMM has been linked to both inherited and non-inherited mechanism based tumor progression. Further investigations addressing this RHAMM link through dc-DEP EMF force in future *in vitro* and *in vivo* models may unravel an important mystery in tumor progression and cancer metastasis. Future trials are now being planned to treat cancer patients with dc-DEP EMF based approaches. The dc-DEP-EMF based therapeutic applications could potentially offer a new adjunct treatment as well as a possible alternative to CPM and other surgical interventions for these patients with inherited and non-inherited aggressive carcinomas if these *in vitro* data prove to be translatable to human clinical trials.

Acknowledgments

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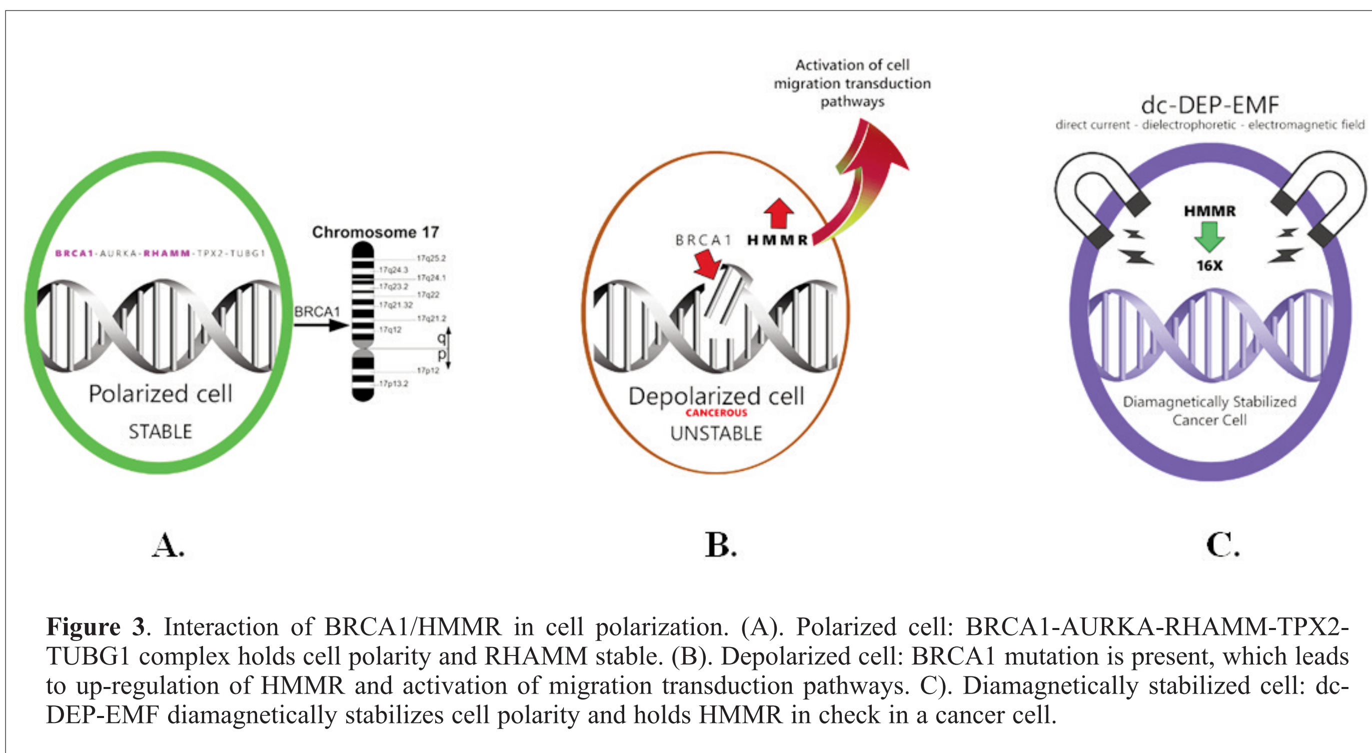

Figure 3. Interaction of BRCA1/HMMR in cell polarization. (A). Polarized cell: BRCA1-AURKA-RHAMM-TPX2-TUBG1 complex holds cell polarity and RHAMM stable. (B). Depolarized cell: BRCA1 mutation is present, which leads to up-regulation of HMMR and activation of migration transduction pathways. C). Diamagnetically stabilized cell: dc-DEP-EMF diamagnetically stabilizes cell polarity and holds HMMR in check in a cancer cell.

committee advisor. The work was conducted in the laboratory of Michael A. Whitt, Ph.D., M.S.B. and Molecular Resource Center at UTHSC. Rita Kansal, Ph.D., helped with technical laboratory support. Elizabeth Tolley, Ph.D., was consulted for statistical support and was a member of my dissertation committee. Terry J. Skrinjar generated Figure 3 under the guidance of Marcy C. Purnell as a paid consultant with the University of Memphis.

Disclosure

M.C.P. is a co-holder of methods/application patent: Declaration (37 CFR 1.63) for Utility or Design (35 USC 111 (a) "Bioelectrodynamics Modulation Method," and the holder of the provisional patent, "Biochloride Generation and Methods" filed with the USPTO in Alexandria, Virginia.

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